Bacterial outer membrane proteins and host mucins involved in colonization of the gastric mucosa by the zoonotic pathogen *Helicobacter heilmannii*

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LIST OF ABBREVIATIONS

AGS	Gastric adenocarcinoma cells
AhpC	Alkyl hydroperoxide reductase
AlpA	Adherence-associated lipoprotein A
AlpB	Adherence-associated lipoprotein B
BabA	Blood group antigen-binding adhesion
BP	bipolar
CagA	Cytotoxin-associated gene A
CagPAI	Cag pathogenicity island
CT	Cytidine-thymidine
DCs	Dendritic cells
DcuA	L-asparaginase
Dmbt1	Deleted in malignant brain tumors 1 protein
DNA	Deoxyribonucleic acid
ECLs	Enterochromaffin-like cells
ECM	Extracellular matrix
E.coli	*Escherichia coli*
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FlaA	Flagellins A
FlaB	Flagellins B
FldA	Flavodoxin protein
FlgE	Flagellar hook
FliD	Flagellar cap
GalNAc	N-acetylgalactosamine
GGT	γ-glutamyl transpeptidase
HBSS	Hank's balanced salt solution
HCL	Hydrochloric acid
Hacin	*H. acinonychis*
Hailuro	*H. ailurogastricus*

Hbac	*H. baculiformis*
Hbiz	*H. bizzozeronii*
Hcet	*H. cetorum*
Hcyn	*H. cynogasricus*
Hfel	*H. felis*
Hh	*H.heilmannii*
H+/K+ ATPase	Hydrogen potassium ATPase
Hmus	*H. mustelae*
Hof	*Helicobacter* OMP family
Hom	*Helicobacter* outer membrane
Hop	*Helicobacter* outer membrane porin
Hor	Hop related
Hp	*H. pylori*
HpaA	*H. pylori* adhesion A
Hsal	*H. salomonis*
Hsuis	*H. suis*
HtrA	High temperature requirement A
IceA	The ulcer-associated protein restriction endonuclease
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL-8	Interleukin-8
ITF	Intestinal trefoil factor
KatA	Catalase
Kb	Kilobase
KCNQ1	Potassium channel, voltage gated KQT-like subfamily Q member 1
kDa	Kilodalton
LacdiNac	N-terminal acetylgalactosamine β1-4N-acetylglucosamine
Le^b	Lewis b blood group antigen
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
MP	monopolar

MUC	Human mucin
Muc	Murine mucin
NapA	Neutrophil-activating protein
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B
NHPH	Non-*Helicobacter pylori Helicobacter* species
NLRP3	Nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3
NOD	nucleotide-binding oligomerization domain
OipA	Outer inflammatory protein A
OMPs	Outer membrane proteins
PAK1	P21-activated kinase 1
PAMPs	Pathogen-associated molecular patterns
PAS	Periodic acid-Schiff
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PH	Measure of acidity or basicity
PIgR	Polymeric immunoglobulin receptor
PRRs	pattern recognition receptors
pS2	presenilin 2 gene
qPCR	Quantitative real-time PCR
rRNA	Ribosomal RNA
RT	Room temperature
SabA	Sialic acid-binding adhesion
SEA	Sea urchin sperm protein enterokinase arginine
sLe^x	Sialylated Lewis x
SodB	Superoxide dismutase
SPEM	Spasmolytic polypeptide-expressing metaplasia
SPF	Specific pathogen free
SSM	Slipped strand mispairing
TFFs	Trefoil factors
Th	T helper
TLRs	Toll-like receptors
TNF	Tumor necrosis factor

T4SS	Type IV secretion system
UreA/B	Urease subunit A/B
VacA	Vacuolating cytotoxin
VNTR	Variable number of tandem repeats
WT	Wild type

REVIEW OF THE LITERATURE

1. The genus *Helicobacter*

Since the description of the human pathogen *Helicobacter pylori* (Warren and Marchal, 1984; Goodwin et al., 1989), the number of known species in the genus *Helicobacter* has been increasingly enlarged. Currently this genus includes more than 40 identified species as shown in Table 1. The *Helicobacter* bacteria can roughly be divided into gastric and enterohepatic species. Gastric *Helicobacter* species are able to survive the acidic environment in the stomach by expressing urease at a high level (Weeks et al., 2000; Pot et al., 2007). Enterohepatic species thrive in the mucosal surfaces of the intestinal tract and/or the liver (Sterzenbach et al., 2007). Several of these animal-associated helicobacters have a pathogenic potential in different animal hosts and some are able to cause disease in humans (Table 1). The *Helicobacteraceae* family has the possibility to expand into new hosts as the intimate contact among different animal species and between animals and humans increases creating the opportunity for interspecies transmission and new strain formation. The presence and diversity of helicobacters in the vertebrate fauna and their transfer possibilities between hosts are critical factors on how fast the *Helicobacter* ecology will evolve and what their impact is on animal and human health (Schrenzel et al., 2010).

Table 1: *Helicobacter* spp. and their hosts (adapted from Flahou et al., 2016)

Taxon	Animal hosts	Zoonotic potential?
Gastric *Helicobacter* spp.		
"Candidatus H. bovis"[a]	cattle	Yes
"Candidatus H. homininae"[a]	chimpanzee, gorilla	Unknown
H. acinonychis	cheetah, tiger, lion	Unknown
H. ailurogastricus	cat	Unknown
H. baculiformis	cat	Unknown
H. bizzozeronii	cat, dog	Yes
H. cetorum	whale, dolphin	Unknown
H. cynogastricus	dog	Unknown
H. felis	dog, cat, cheetah, new Guinea wild dog, rabbit	Yes
H. heilmannii	dog, cat, cheetah, bobcat, tiger, lynx leopard, puma	Yes
H. mustelae	ferret	Unknown
H. pylori	human	/
H. salomonis	cat, dog, rabbit	Yes
H. suis	pig, mandrill monkey, rhesus macaque, crabeating macaque	Yes
Taxon	**Natural hosts**	**Zoonotic potential?**
Enterohepatic *Helicobacter* spp.		
"Candidatus H. colifelis"[a]	cat	Unknown
H. anseris	goose	Unknown
H. aurati	hamster	Unknown
H. bilis	mouse, rat, gerbil, dog, cat, sheep	Yes
H. brantae	goose	Unknown
H. callitrichis	marmoset	Unknown
H. canadensis	bird, pig	Yes

H. canis	dog, cat	Yes
H. cholecystus	hamster	Unknown
H. cinaedi	hamster, rat, cat, dog, rhesus monkey, baboon	Yes
H. equorum	horse	Unknown
H. fennelliae	dog	Yes
H. ganmani	mouse	Yes
H. hepaticus	mouse, gerbil	Yes
H. macacae	rhesus monkey, baboon	Unknown
H. marmotae	woodchuck, cat	Unknown
H. magdeburgensis	mice	Unknown
H. mastomyrinus	rodents	Unknown
H. mesocricetorum	hamster	Unknown
H. muricola	wild mouse	Unknown
H. muridarum	mouse, rat	Unknown
H. pamatensis	bird, pig, cat	Yes
H. pullorum	poultry	Yes
H. rappini	mouse, sheep, dog	Yes
H. rodentium	mouse, rat	Unknown
H. sanguine	cotton-top tamarin	Unknown
H. suncus	house musk skrew	Unknown
H. trogontum	rat, pig, sheep	Unknown
H. typhlonius	mouse, rat	Unknown
H. valdiviensis	wild birds	Unknown
H. westmeadii	human	/
H. winghamensis	human, wild rodents	Yes

[a] These *Helicobacter* species have not yet been isolated and cultivated *in vitro*.

2. Gastric *Helicobacter* species in humans and animals

H. pylori is the most prevalent *Helicobacter* species in the stomach of humans and has been considered as one of the most successful human pathogens. This slightly-curved microorganism has been associated with a wide range of gastric disorders including, gastritis, peptic ulcer disease, mucosa-associated lymphoid tissue (MALT) lymphoma and cancer (Parsonnet et al., 1991; Stolte et al., 1993; Kusters et al., 1996; Ishaq & Nunn, 2015).

It is estimated that half of the human population in the world is infected with *H. pylori* albeit with large geographical variations. In developing countries, more than 80% is infected with *H. pylori*, whereas the prevalence rate of *H. pylori* in more developed countries varies from 20 to 50% (Kusters et al., 2006; Pounder and Ng, 1995). This variation in prevalence can be explained by a combination of factors, such as the age at acquisition of infection, the type of *H. pylori* strain, the genetic profile and socioeconomic status of the host as well as environmental factors. *H. pylori* infections are frequently acquired during early childhood and the disease progresses with age (Suerbaum and Josenhans, 2007).

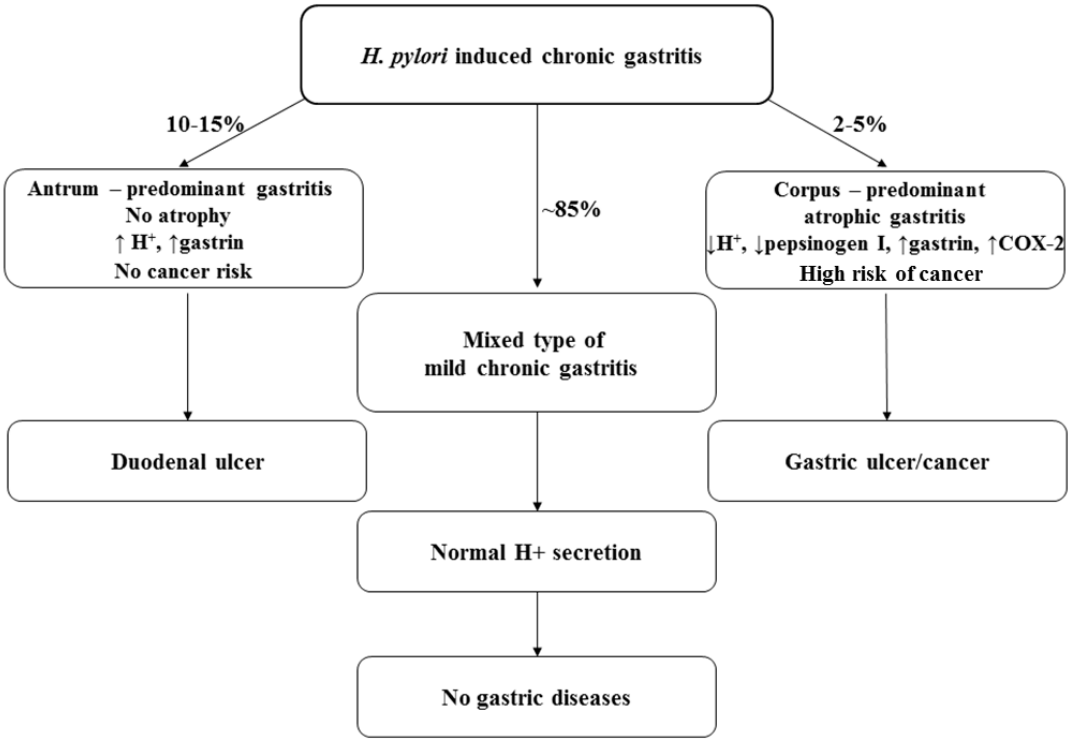

Figure 1: Sequential steps of diseases resulting from *H. pylori* infection (Karczewska et al., 2014). The percentages of *H. pylori*-infected people developing a certain gastric pathology are indicated as well. Gastritis is localized mainly in the antral portion of the stomach (antrum-predominant gastritis) or potentially extending to the gastric corpus (corpus-predominant gastritis). There is a clear interaction between *H. pylori*-associated gastritis and gastric acid secretion. Predominant antral gastritis is associated with an increase in acid secretion and predisposes for duodenal ulceration. Patients with duodenal ulcers almost never develop gastric cancer. This finding may suggest that duodenal ulcers somehow provide a protective mechanism against gastric malignancy (Konturek et al., 2006; Figure 1). On the contrary, corpus-predominant atrophic gastritis is accompanied with a decrease in acid secretion. Humans showing this latter pathology have an increased risk to develop gastric cancer. The predisposition for developing gastric adenocarinoma involves the interaction of three major factors: the virulence of the pathogenic agent, the host immune system and environmental factors.

Most of the *H. pylori* infections in humans are asymptomatic and only 10-20% of the people carrying *H. pylori* will develop gastric symptoms later on in their lives (Kusters et al., 2006; Suerbaum and Josenhans, 2007). A schematic overview explaining the different gastric disease outcomes in humans induced by *H. pylori* infection is shown in Figure 1.

However, *H. pylori* is not the only *Helicobacter* species causing gastric disease in humans. In the early years of *H. pylori* research, pathologists examining human gastric biopsies reported the presence of bacteria with a long spiral-shaped morphology. These microorganisms were similar to bacteria earlier reported in the stomach of pigs, cats, dogs and non-human primates (Salomon, 1898; Mendes et al., 1990; Queiroz et al., 1990) and have been found in 0.2 to 6% of gastric biopsies from humans with severe gastric complaints (Heilmann and Borchard, 1991; Stolte et al., 1994; Svec et al., 2000; Solnick et al., 2003). They were originally referred to as "*Gastrospirillum hominis*" (McNulty et al., 1989). Analysis of the 16S rRNA gene of these non-*H. pylori Helicobacter* spp. (NHPH) resulted in their classification into the genus *Helicobacter*. They were renamed "*H. heilmannii*" after the German pathologist Konrad Heilmann who first studied the pathology associated with these helicobacters (Heilmann&Borchard, 1991). Although the name *H. heilmannii* had at that time no official standing in nomenclature, for many years it was used to refer to the whole group of long spiral-shaped bacteria in the human stomach, which actually comprise several different *Helicobacter* species.

2.1. Gastric non-*H. pylori Helicobacter* nomenclature, characteristics and phylogeny

As a sequel to the renaming of "*Gastrospirillum hominis*" as "*H. heilmannii*", further analysis of the 16S rRNA gene sequence resulted in the reclassification of these gastric helicobacters into "*H. heilmannii*" type 1 and "*H. heilmannii*" type 2. These 2 types differ by more than 3% in nucleotide sequence of the 16S rRNA gene. "*H. heilmannii*" type 1 is identical to *H. suis*, which naturally colonizes the stomach of pigs (Baele et al., 2008b; De Groote et al., 1999; O'Rourke et al., 2004b). *H. heilmannii* type 2 is more complex. It does not represent one single species, but rather a group of species naturally colonizing the canine and feline gastric mucosa, such as *H. bizzozeronii*, *H. felis*, *H. salomonis*, *H. cynogastricus*, *H. baculiformis*, *H. heilmannii* and *H. ailurogastricus* (Table 1; Haesebrouck et al., 2009; Smet et al., 2011; Baele et al., 2008a; Van den Bulck et al., 2006; Joosten et al., 2015). For *H. ailurogastricus*, *H. cynogastricus* and *H. baculiformis*, no information is available about their zoonotic potential in humans. The valid description of *H. heilmannii* as a species further added to the confusion

on the nomenclature of this complex and expanding group of microorganisms (Smet et al., 2011). Therefore, the terms *H. heilmannii* (sensu lato), referring to the group of gastric NHPH, and *H. heilmannii* (sensu stricto), referring to the species, have been introduced (Haesebrouck et al., 2011).

Although the porcine, feline and canine *Helicobacter* spp. all have a typical spiral shape, differences in morphology between these NHPH have been described. An overview is shown in Table 2. *H. felis, H. cynogastricus* and *H. baculiformis* possess periplasmic fibrils (Eaton et al., 1996; Van den Bulck et al., 2006, Baele et al., 2008a), while *H. bizzozeronnii, H. heilmannii, H. ailurogastricus, H. salomonis* and *H. suis* do not have these structures (Hänninen et al., 1996; Jalava et al., 1997; Baele et al., 2008b, Smet et al., 2011; Joosten et al., 2015). *H. felis, H. cynogastricus, H. bizzozeronii, H. heilmannii, H. ailurogastricus* and *H. suis* are known to have very tight coils, whereas *H. baculiformis* and *H. salomonis* are rather slender, slightly spiral-shaped rods. These bacteria also differ from each other in cell length, number of spiral turns and bipolar flagella and their ability to hydrolyze biochemical substrates (Table 2).

Besides the canine, feline and porcine gastric helicobacters, 3 other animal-associated *Helicobacter* spp., with a morphology more similar to that of *H. pylori*, have been described in the early 90s and the beginning of the 21st century. These include *H. acinonychis* from wild felines, *H. cetorum* from marine mammals and *H. mustelae* from ferrets (Paster et al., 1991; Harper et al., 2002; Eaton et al., 1993). Their presence in humans has so far not been described. An overview of the morphological and biochemical characteristics of *H. acinonychis, H. cetorum* and *H. mustelae* is presented as well in Table 2.

Table 2: Morphological and biochemical characteristics of gastric *Helicobacter* species.

	Hh	Hailuro	Hfel	Hbiz	Hsal	Hcyn	Hbac	Hsuis	Hp	Hacin	Hcet	Hmust
Cell length (µm)	3-6.5	3-5.5	5-7.5	5-10	5-7	10-18	10	2.3-6.7	2.5-5.0	1.5-2	4	2.5-5
Cell width (µm)	0.6-0.7	0.5-0.7	0.4	0.3	0.8-1.2	0.8-1.0	1	0.9-1.2	0.5-1.0	0.3	0.6	0.5
Urease activity	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate production	+	+	+	+	+	+	+	-	-	-	-	+
Alkaline phosphatase activity	-	+	+	+	+	+	+	+	+	+	-	+
Hydrolysis of indoxylacetate	-	-	-	+	+	-	-	-	-	-	-	+
Periplasmic fibril	-	-	+	-	-	+	+	-	-	-	-	-
Growth at 42°C	-	-	-	+	-	-	-	-	-	-	+	+
Flagella												
No. per cell	4-10	6-8	14-20	10-20	10-23	6-12	11	4-10	4-8	2-5	1	4-8
Distribution	BP	BP	BP	BP	BP	BP	BP	BP	MP	MP	BP	peritrichous

Hh = *H. heilmannii* (Smet et al., 2011), Hailuro = *H. ailurogastricus* (Joosten et al., 2015), Hfel = *H. felis* (Hänninen et al., 1996; Lee et al., 1988; Jalava et al., 1997), Hbiz = *H. bizzozeronii* (Hänninen et al., 1996), Hsal = *H. salomonis* (Jalava et al., 1997), Hcyn = *H. cynogastricus* (Van den Bulck et al., 2006), Hbac = *H. baculiformis* (Baele et al., 2008a), Hsuis = *H. suis* (Baele et al., 2008b), Hp = *H. pylori* (Hänninen et al., 1996; Jalava et al., 1997), Hacin = *H. acinonychis* (Eaton et al., 1993); Hcet = *H. cetorum* (Harper et al., 2002), Hmust = *H. mustelae* (Paster et al., 1991). BP = bipolar, MP = monopolar. Urease, catalase, oxidase and γ-glutamyltranspeptidase activity was uniformly present; growth in the presence of 1% glycine was uniformly absent. Gastric *Helicobacter* spp. require a highly humidified micro-aerobic environment, consisting of 10% CO_2, 5% O_2 and 85% N_2.

Despite the difference in morphology among the gastric NHPH, microscopic investigation of biopsy samples is not an accurate method for species identification (Eaton et al., 1996).

Analysis of the 16S rRNA gene sequence has frequently been used in the past for differentiation between gastric *Helicobacter* species (O'Rourke et al., 2004b). Though, sequencing of the 16S rRNA cannot distinguish between *H. bizzozeronii*, *H. felis*, *H. salomonis*, *H. baculiformis*, *H. cynogastricus* and *H. heilmannii* (Smet et al., 2011; Baele et al., 2008a; Van den Bulck et al., 2006; Dewhirst et al., 2005). For differentiation between those species, analyses of the urease A and B and the *hsp60* gene sequences have been performed. The similarity of the *ureAB* genes from the canine and feline gastric helicobacters is lower than 85%, which allowed discrimination between these species (O'Rourke et al., 2004b). However, it has been shown that the recently described *H. ailurogastricus* species from cats could not be distinghuised from its closest relative *H. heilmannii* by means of its 16S rRNA and *ureAB* genes. This implicates that the discriminatory capacity of these gene sequences is not high enough for the distinction of closely related gastric *Helicobacter* species. In this respect, genome sequencing based approaches are superior compared to the traditional 16S rRNA sequence analysis for studying phylogeny because they are based on the complete genome content and have a better resolution for discriminating both distantly and closely related bacteria (Vandamme, 2014). A phylogenetic tree based on the core genomes of all gastric *Helicobacter* spp. described so far is shown in Figure 2.

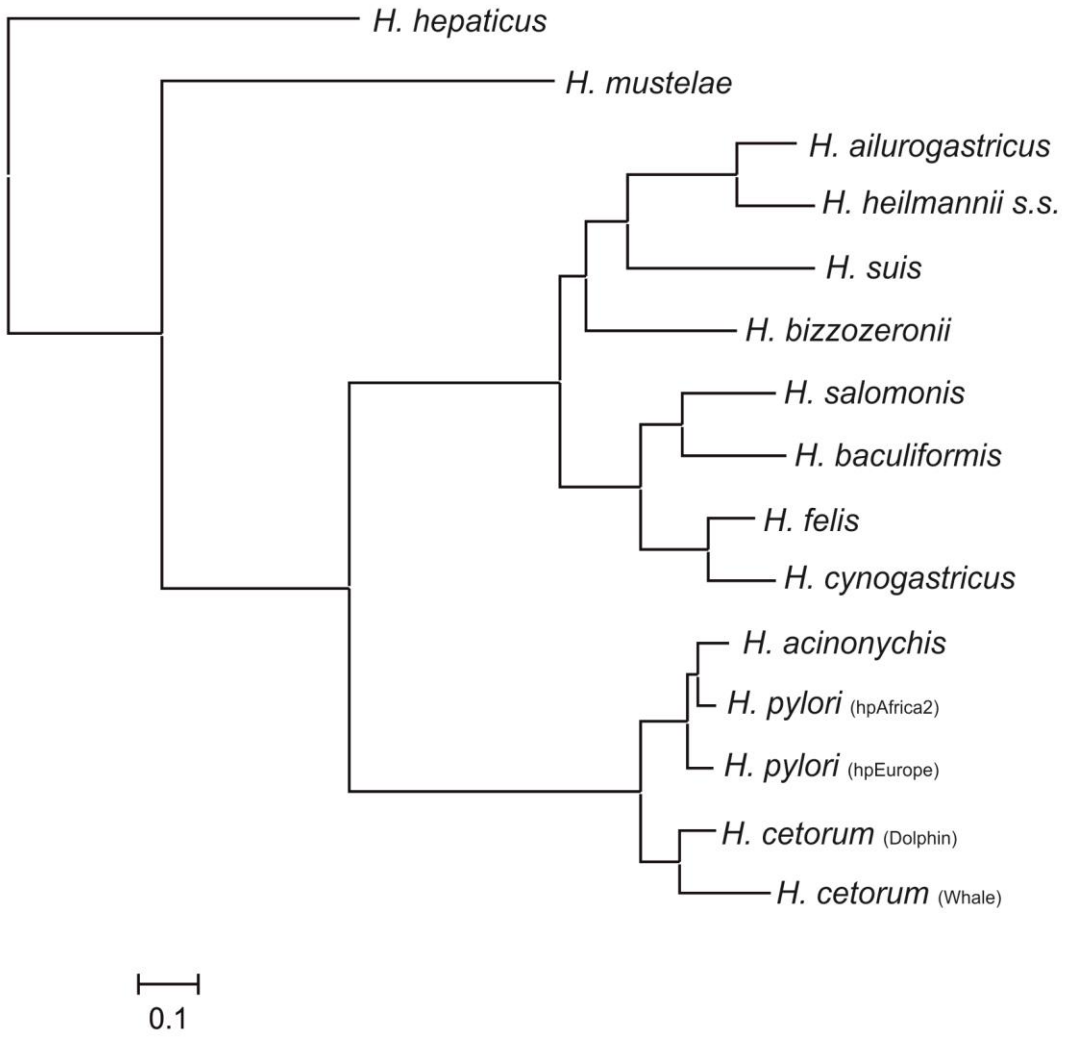

Figure 2: A phylogram representing the maximum-likelyhood tree of gastric *Helicobacter* spp. based on aligned and concatenated core genes. The enterohepatic *H. hepaticus* species was used as outgroup. Three gastric cluster can be seen. One cluster comprises the porcine, canine and feline helicobacters, one comprises *H. mustelae* and one comprises *H. pylori, H. acinonychis* and *H. cetorum* (unpublished results).

2.2. Animal-associated gastric *Helicobacter* infections in humans: clinical significance

To date, evidence is accumulating that domestic animals constitute a reservoir for gastric *Helicobacter* spp. with zoonotic potential. A significant number of reports has revealed that people who have close contact with dogs, cats and pigs are more likely to be infected with an animal-associated gastric *Helicobacter* species, such as *H. heilmannii* (Chung et al., 2014; De Bock et al., 2007; Joosten et al., 2013; Meining et al., 1998). *Helicobacter* DNA has been detected in saliva from cats, dogs and pigs indicating that the oral cavity of these animals may

act as source of NHPH infection for humans (Ekman et al., 2013; Casagrande Proieti et al., 2010; ShojaeeTabrizi et al., 2010). Fecal-oral transmission has also been suggested as a possible route for infection in cats (Ghil et al., 2009). Besides direct contact with animals, other routes of transmission of NHPH should not be neglected. It has been shown that *H. felis* is able to survive in water for several days highlighting the possible role for water in the transmission of this species (Azevedo et al., 2008). *H. suis* can be present on and survive in minced pork. This indicates that raw or undercooked pork may also constitute a source of *H. suis* infection for humans (De Cooman et al., 2013).

H. suis, *H. heilmannii*, *H. bizzozeronii*, *H. felis* and *H. salomonis* (Table 1) have been associated with gastritis, gastric and duodenal ulcers and low grade MALT lymphoma in humans (Haesebrouck et al., 2009; Matsumoto et al., 2014; Iwanczak et al., 2012; Duquenoy and Le Luyer, 2009). These NHPH have microscopically been detected in 0.2-6% of humans with severe gastric complaints undergoing a gastroscopy. This is most probably an underestimation of their true prevalence since it cannot be excluded that infections with these agents sometimes remain unapparent or cause mild disease signs which are not often thoroughly examined (Haesebrouck et al., 2009). Also they are not that easily detected in gastric biopsy samples by microscopical examination since their colonization pattern is much more focal and patchy than that of *H. pylori*. So examination of a small biopsy sample will not always reveal the presence of spiral-shaped bacteria in infected human patients (Haesebrouck et al., 2009; Trebesius et al., 2001; De Groote et al., 2005; Iwanczak et al., 2012; Yakoob et al., 2012). Interestingly, the prevalence of *H. pylori* in humans from the Western World is decreasing from generation to generation (Bhandari and Crowe, 2012), leaving a niche for possible infection with these animal-associated gastric *Helicobacter* spp.

Clinical symptoms associated with gastric NHPH infections include acute or chronic epigastric pain, nausea, dyspepsia, reflux esophagitis, heartburn, vomiting, hematemesis, abdominal pain, irregular defecation frequency and consistency and dysphagia, often accompanied by a decreased appetite (Dieterich et al., 1998; Goddard et al., 1997; Heilmann&Borchard, 1991; Iwanczak et al., 2012; Joosten et al., 2013; Matsumoto et al., 2014; Mention et al., 1999; Oliva et al., 1993; Solnick&Schauer, 2001).

In patients undergoing an endoscopy, a variety of lesions can be observed ranging from a normal to slightly hyperaemic mucosa, mucosal oedema, nodular inflammation and the presence of ulcerations in the antrum of the stomach or in the duodenum (Haesebrouck et al., 2009; Sykora et al., 2003; Van Loon et al., 2003; Yang et al., 1995; Yoshimura et al., 2002).

Histological examination of gastric biopsies is generally characterized by infiltration with lymphocytes and plasma cells. In some cases, lymphocytic aggregates or intestinal metaplasia have been described (Ierardi et al., 2001; Joosten et al., 2013; Matsumoto et al., 2014; Morgner et al., 2000; Yakoob et al., 2012). Compared to an *H. pylori*-associated gastritis, gastritis associated with NHPH is often less active and less severe. On the other hand, the risk of developing MALT lymphoma is higher with NHPH than with *H. pylori* (Haesebrouck et al., 2009; Matsumoto et al., 2014; Joosten et al., 2013).

The inflammatory effects caused by these NHPH seem not only to be restricted to the stomach. An exceptional high frequency (27%) of *H. suis* DNA was found in gastric biopsies from human patients with Parkinson's disease compared to a control group with no clinical parkinsonism. Besides in the stomach, in our research group, *H. suis* DNA was detected in a blood sample of a patient with parkinsonism who was also diagnosed with Alzheimer's disease. This patient received a triple therapy of tetracyclines, clarithromycin and a proton pump inhibitor to eradicate *H. suis.* Interestingly, the *H. suis* infection was cleared and the gastric and neurological symptoms improved remarkably (Blaecher et al., 2013). Subsequently to these latter findings, an *in vivo H. suis* infection study in mice highlighted the potential role of *H. suis* infection in neuro-inflammation (Blaecher C., 2015, personal communication).

Commercial tests to diagnose infection with gastric NHPH are currently unavailable. Urea breath tests, used to diagnose infection with *H. pylori*, are often negative in patients infected with animal-associated gastric *Helicobacter* spp., such as *H. heilmannii* (Matsumoto et al., 2014). This can be explained by the fact that infections with these bacteria are, in contrast with *H. pylori* infections, more often focal and predominantly found in the antrum of the stomach (Solnick and Schauer, 2001). Only a low number of animal-associated gastric *Helicobacter* isolates are available worldwide. Due to their fastidious nature, isolation of these bacteria is not an option for routine diagnostic purposes. So far, 2 gastric *Helicobacter* species, namely *H. bizzozeronii* (Andersen et al., 1999; Kivisto et al., 2010) and *H. felis* (Wüppenhorst et al., 2013), have been cultured from gastric biopsies taken from infected human patients. Therefore, analysis of gastric biopsies by molecular microbiological methods and histology is at this moment the only way to determine infections with these pathogens (Haesebrouck et al., 2009).

For patients with severe gastric complaints and evidence of an NHPH infection, treatment is necessary. There is, however, a lack of clinical trials and only few reports deal with

antimicrobial susceptibility tests of gastric NHPH (Vermoote et al., 2011; Van den Bulck et al., 2005a; Blaecher et al., 2013). Triple therapy using the combination of a proton pump inhibitor and two antimicrobial agents, like clarithromycin, metronidazole, amoxicillin or tetracycline may be effective in most cases but not always due to the presence of acquired resistance against antimicrobial agents among these gastric helicobacters (Schott et al., 2012; Kondadi et al., 2013).

2.3 *Helicobacter heilmannii* in domestic pets and wild felines

2.3.1 Prevalence of *H. heilmannii*

H. heilmannii, formerly "*Candidatus* H. heilmannii" (O'Rourke et al., 2004a; Smet et al., 2012), naturally colonizes the stomach of domesticated cats and dogs (Haesebrouck et al., 2009; Smet et al., 2012). According to the literature, this microorganism has more frequently been detected in cats than in dogs (Smet et al., 2012; Takemura et al., 2009; Canejo-Teixeira R et al., 2014). This bacterium has also occasionally been found in the stomach of wild felines, including lynx, leopards, pumas, bobcats, tigers and cheetahs (Hamir et al., 2004; Mörner et al., 2008; Terio et al., 2005; O'Rourke et al., 2004a; Luiz de Camargo et al., 2011). The prevalence of NHPH ranges from 20% to 100% in the gastric mucosa of healthy pets as well as animals showing chronic vomiting and other symptoms of gastrointestinal discomfort (Haesebrouck *et al.*, 2009; Hwang *et al.*, 2002; Neiger *et al.*, 1998; Van den Bulck*et al.*, 2005a; Amorin et al., 2015). Additionally, pet animals infected with *H. heilmannii* can also be co-infected with an additional gastric *Helicobacter* species, e.g. *H. felis* (Haesebrouck et al., 2009; Smet et al., 2012).

2.3.2 Pathogenic significance of *H. heilmannii* in its natural hosts and experimentally infected lab animals

In general, *H. heilmannii* has been associated with chronic active gastritis in pet animals (Haesebrouck et al., 2009). Histological changes in the lamina propria including mild mononuclear inflammatory infiltration, the presence of lymphoid follicles, fibrosis and glandular degeneration have been described in the stomach of cats naturally infected with *H. heilmannii* (Takemura et al., 2009). In cheetahs, gastritis caused by *H. heilmannii* was characterized by the infiltration of lymphocytes and plasma cells in the epithelium and lamina propria with gland destruction and parietal cell loss. In some cases lymphoid follicles were observed especially in captive animals and less frequently in wild animals (Terio et al., 2005;

Munson et al., 2005). Terio et al. (2012) further investigated the local gastric immune response in cheetahs with varying degree of *Helicobacter*-associated gastritis. The type of cells involved in the host immune response was similar among all types of gastritis with the exception that a large number of lamina proprial activated $CD79a^{+}CD21$-B cells and plasma cells were only seen in cheetahs with severe gastritis (Terio et al., 2012).

To study the pathogenesis of a gastric *H. heilmannii* infection, experimental infection studies in specific pathogen free (SPF) laboratory animals have been performed (O'Rourke et al., 2004a; Joosten et al., 2013; Nakamura et al., 2014). Rodent animals have been shown to be useful models for the study of *Helicobacter*-related gastric disease (O'Rourke and Lee, 2003; Rogers and Fox, 2004). *H. heilmannii* has been propagated in mice for up to 28 months and was able to induce MALT lymphoma in the stomachs of these animals (O'Rourke et al., 2004a). However in this mouse experiment, homogenized animal gastric tissue was used as inoculum. This implies that other microorganisms were inoculated together with *H. heilmannii*, which might have influenced the results (Flahou et al., 2010). Thus, to obtain better insights into the pathogenicity of *H. heilmannii*, experimental infection studies with pure cultures of this microorganism are essential. A recent infection study with pure feline *H. heilmannii* strains, using a Mongolian gerbil model, showed an antrum-dominant chronic active gastritis with formation of large lymphocytic aggregates in the stomach of animals infected for 9 weeks (Joosten et al., 2013).

Although infection with *H. heilmannii* has been associated with the development of severe gastric pathologies in its natural hosts, its clinical significance in those animals still remains not completely understood and is most probably strain-dependent or may be related to host differences (Haesebrouck et al., 2009).

3. The gastric mucosa: a niche for *Helicobacter*

3.1 Anatomy and physiology of the human gastric mucosa

The gastric mucosa is the layer of the stomach which contains the glands and the gastric pits. It consists of the epithelium, lamina propria and muscularis mucosa (Eurell and Frappier, 2006; Figure 3).

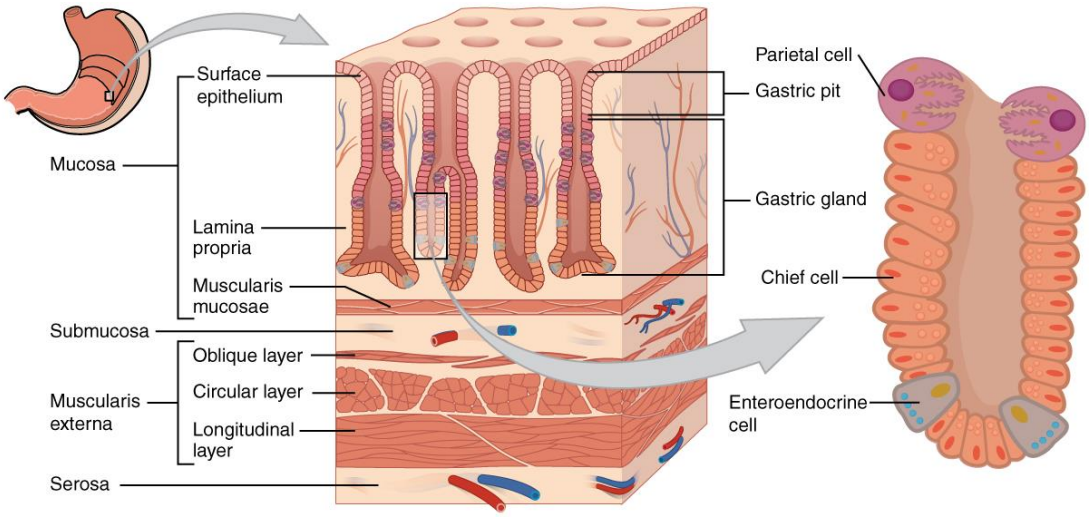

Figure 3: Schematic representation of the human gastric mucosa. In the epithelium, gastric pits lead to gastric glands that secrete gastric juice. The gastric glands (one is shown enlarged on the right), showing a simple or branched tubular morphology, contain different cell types that secrete a variety of substances, including hydrochloric acid (HCl), which activates the protein-digesting enzyme pepsin (adapted from Open Stax College, Anatomy and Physiology. OpenStax CNX. http://cnx.org/contents/14fb4ad7-39a1-4eee-ab6e-3ef2482e3e22@8.24)

The surface epithelium and the gastric pits of the gastric mucosa are made up primarily of mucous cells, which secrete a coat of mucus that functions as a protective barrier against pathogens (Figure 3).

On the other hand, the gastric glands consist of different cell types (Figure 3 & 5). According to the different zones in the stomach, there are 3 types of gastric glands (Figure 4): cardiac glands (in the proximal part of the stomach), fundic (oxyntic) glands (the dominating type of gland), and pyloric glands (in the antrum of the stomach).

The glands of the cardia and pylorus are composed primarily of mucus-secreting cells. The pyloric antrum additionally contains enteroendocrine cells secreting hormones, such as gastrin (G-cells) and somatostatin (D-cells) (Soybel, 2005). The glands of the fundus and the body of the stomach (Figures 4&5), produce most of the gastric secretions and are made up of a variety of secretory cells. These include HCl-secreting parietal cells, zymogen (pepsinogen)-secreting chief cells, mucous neck cells, enterochromaffin-like cells and enteroendocrine cells (Figures 3 & 5). Below an overview of the main function of these cells is presented.

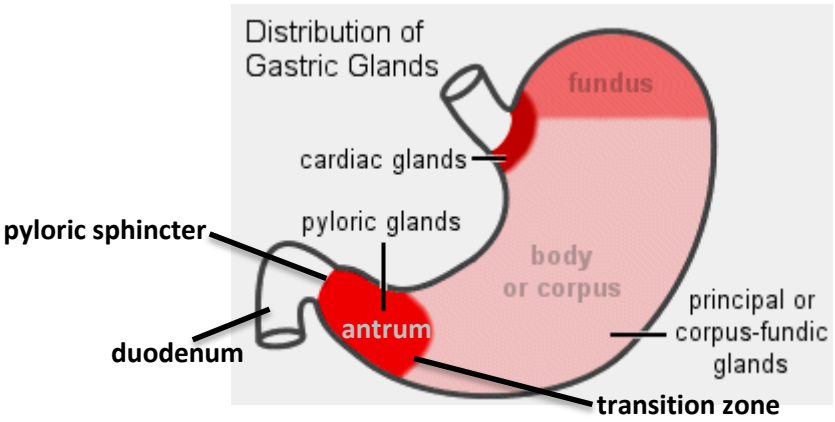

Figure 4: Schematic representation of the different gland regions (pyloric, fundic, and cardiac glands) in the human stomach. The fundic gland region and the pyloric gland region can be distinguished by the transition zone and the pylorus can be easily palpated, with a ring of muscle (pyloric sphincter) separating the stomach and the duodenum.

Parietal cells contain an extensive secretory network named canaliculi, from which the HCl is secreted by active transport into the gastric lumen (Figures 3&5). When stimulated, these cells massively secrete H^+ into the gastric lumen, at a concentration of roughly 160 mM, which is equivalent to a pH of 0.8. Acid facilitates the activation of pepsinogen, the digestion of food and absorption of iron, calcium, and vitamin B-12, and it also controls bacterial overgrowth and enteric infection (Schubert et al., 1990). The ability of the parietal cell to secrete acid is dependent on active transport. The H^+, K^+ -ATPase of parietal cells is the proton pump composed of a catalytic subunit (α-subunit) and an accessory subunit (β-subunit). The dephosphorylation reaction of mass adenosine triphosphate (ATP) releases energy. So the H^+, K^+ -ATPase transports protons against a huge gradient into the gastric lumen in exchange for K^+ (Yao et al., 2003; Figure 5). **Chief cells** secrete pepsinogen, the inactive proenzyme form of pepsin. HCl is necessary for the conversion of pepsinogen to pepsin. **Mucous neck cells** secrete thin acidic mucus which is different from the alkaline mucus released by the cells at the surface epithelium. **Enterochromaffin-like cells (ECLs)** produce histamine, which is a powerful stimulant of acid secretion (Yao et al., 2003; Figure 5). The hormone gastrin is released by **enteroendocrine G-cells** and also stimulates acid production (Figure 5).

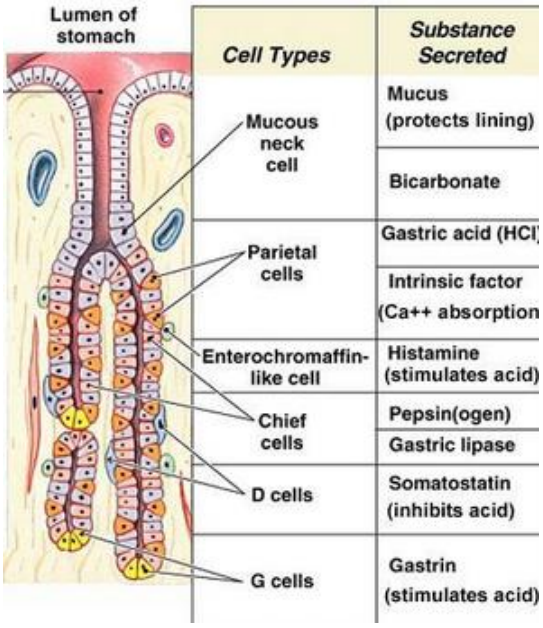

Figure 5: Schematic representation of a gastric fundic gland comprising different epithelial cell types. (Adapted from https://www.studyblue.com/notes/note/n/37-secretion-in-gi-system/deck/11297865)

The fundic gland region and the pyloric gland region can be distinguished by the transition zone from obliquely oriented rugae (internal surface of the stomach) to a relatively flat mucosa, and the pylorus can be easily palpated, with a ring of muscle separating the stomach and the duodenum (Soybel, 2005; Figure 4).

3.2 The mucus layer covering the healthy human gastric mucosa: mucins (MUCs) and trefoil factors (TFFs)

The mucus layer is the first barrier pathogens encounter and mainly consists of high molecular mass oligomeric secreted mucins (MUCs) and trefoil factors (TFFs) (Figure 5; Kaneko et al., 2003; Chen et al., 2004; Lindén et al., 2002; McGuckin et al., 2011; Ringel and Lohr, 2003; Kawakubo et al., 2004). Secreted mucins are produced by cells in the epithelial cell surface and/or the glands. Small amounts of mucins are continuously secreted to maintain the mucus layer whereas high amounts of mucins will be released as a response to environmental and/or (patho)physiological stimuli. The membrane-associated mucins, located underneath the mucus layer, also provide a barrier, thereby limiting access to the cell surface, as well as a reporting function, due to their cytoplasmic tail with signalling potential (McGuckin et al., 2011; Atuma et al., 2001). In the human stomach but also among several mammalian species, including pigs, cats and dogs, MUC1, MUC5AC and MUC6 are the major mucins covering the healthy gastric mucosa (Karlsson et al., 1997; Lacunza et al.,

2009). The membrane-associated MUC1 is expressed at the surface epithelium. The secreted MUC5AC is also restricted to the surface epithelium, whereas the secreted MUC6 is expressed by the glands (Lindén et al., 2002; McGuckin et al., 2011). In addition to the membrane-bound form of MUC1, secreted variants of this mucin have also been reported. MUC5AC and MUC6 are large oligomeric mucins that occur as distinct glycoproteins.

The intestinal mucins MUC2, MUC4, MUC13 and MUC5B are normally not expressed in the human gastric mucosa but their presence has been described in gastric cancer (Ho et al., 1995; Pinto-de-Sousa et al., 2004; Babu et al., 2006). MUC2 is the main secreted mucin making up the mucus layer in the intestines. MUC4 is a cell surface mucin and is mainly expressed in normal colon tissue. The transmembrane MUC13 is expressed in the small and large intestine whereas MUC5B is the predominant mucin in the human gallbladder and in a subset of colonic goblet cells (Van Klinken et al., 1998).

Mucins contain in their nucleotide sequence unique tandem repeat motifs coding for regions with a high density of serine, threonine and proline. The tandem repeats can vary in length between mucins and a genetic polymorphism in the number of repeats, also called Variable Number of Tandem Repeats (VNTR) polymorphism, has been described. Serine and threonine can be *O*-glycosylated and most of the mucin molecular mass is due to the presence of carbohydrate structures (Figure 6).

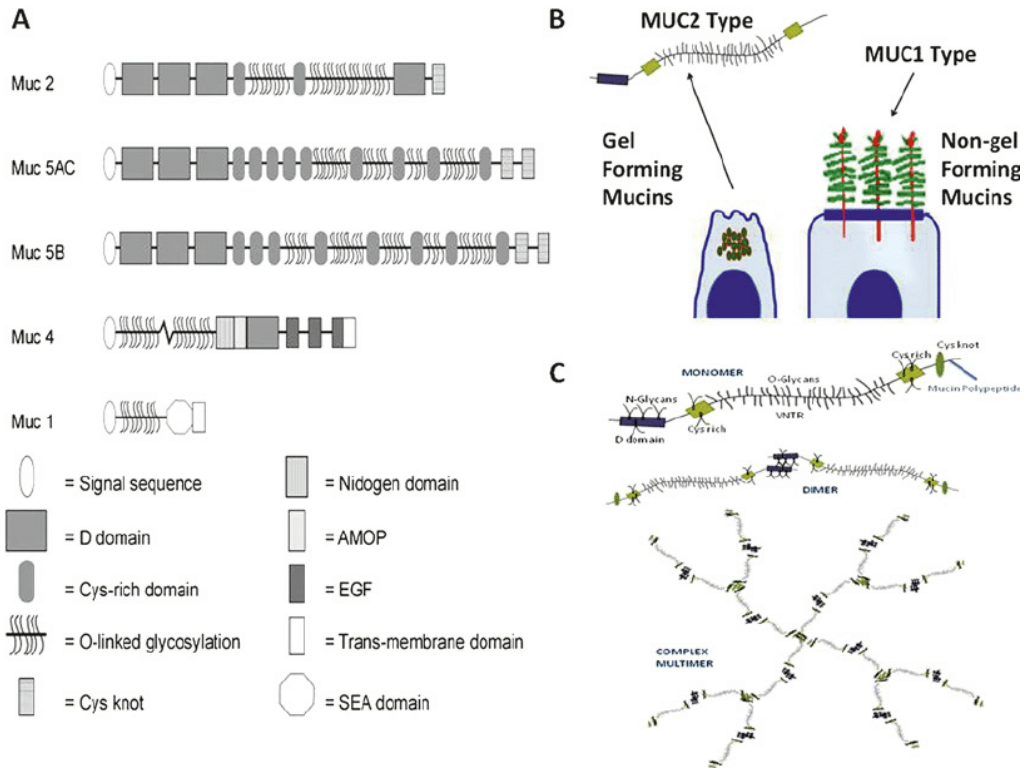

Figure 6: Mucin structure and glycosylation. (A) Schematic representation of structures of secreted and cell-membrane-associated mucins showing different domains. (B) Cartoon showing distribution and structure of gel-

forming secreted, non-gel-forming secreted, and membrane-bound mucins. (C) Schematic representation of the complex glycosylated structure (bottle-brush appearance) and multimeric assembly of a gel-forming secreted mucin. Immediately after its synthesis and translocation into the endoplasmic reticulum, the mucin polypeptide chain is N-glycosylated and forms disulfide-bonded dimers through its C-terminal Cys-rich domains. The dimers are then transported to the Golgi complex, and the tandem repeat domains are O-glycosylated. Once the glycosylated dimers reach the trans-Golgi compartments, they are assembled into disulfide-bonded multimers through their N-terminal D-domains. The Y-shaped structures on the N-terminal D-domains and the C-terminal Cys-rich domains represent N-linked oligosaccharides. The squiggly lines perpendicular to the polypeptide chain represent O-linked oligosaccharides in the tandem repeat domains (adapted from Moran et al., 2011).

Mucins can carry on the order of 100 different carbohydrate structures, which provide the mucins with a bottle-brush appearance and makes them act as receptors for microorganisms (Figure 6C; Klein et al., 1993).

The carbohydrate structures contain an *N*-acetylgalactosamine (GalNAc) group linked to serine or threonine and are elongated by the formation of core structures, followed by type-1 or type-2 chains. The chains can be terminated by fucose (Fuc), galactose (Gal), GalNAc, *N*-acetylglucosamine (GlcNAc) or sialic acid residues in the peripheral region, which form histo-blood-group antigens such as A, B, H, Lewis (Le)^{a}, Le^{b}, Le^{x}, Le^{y}, sialyl(s)Le^{a} and sLe^{x}. Sulfation of Gal and GlcNAc causes further diversification (McGuckin et al., 2011). In the human stomach, Le^{a} and Le^{b} are expressed by MUC1 and MUC5AC, and Le^{x} and Le^{y} by MUC6 (Lindén et al., 2002; Lindén et al., 2009). It should be noted that carbohydrate structures present on mucins differ between people with different histo-blood groups (Marionneau et al., 2001). Mucins from a healthy human stomach express low levels of sialic acid and sulfate and are therefore predominantly neutral.

In humans, three TFFs, namely TFF1/presenilin (pS2), TFF2/spasmolytic polypeptide (SP), and TFF3/intestinal trefoil factor (ITF) are known. Trefoil factors are small, soluble peptides with trefoil or P domains. The trefoil domains are made up of six cysteine residues (Hoffmann and Hauser, 1993; Katoh, 2003). TFFs are secreted from the granules of mucus-secreting cells and are cross-linking with mucins to help form the gel layer in the stomach (Shirazi et al., 2000; Clyne et al., 2004; Julia et al., 2009). TFF1 is normally found in the superficial cells of the body and antral mucosa of the stomach, whereas TFF2 is found in the mucus neck cells of the body and antral glands in the stomach. TFF3 is normally not expressed in the stomach but is expressed in the intestines and the salivary glands (Wong et al., 1999). Previous studies in humans have shown that TFF1 interacts with MUC5AC, TFF2 interacts with MUC6 and

TFF3 interacts with MUC2 (Clyne et al., 2004; Ruchaud-Sparagano et al., 2004; Thim and May, 2005).

3.3 Mucin dynamics associated with *Helicobacter*-induced gastric pathologies

Most mucins exhibit considerable genetic polymorphism due to variability in their numbers of tandem repeat peptides, which results in proteins of widely divergent lengths. The mucin MUC1 is constitutively expressed by the gastric mucosa and is likely the first point of contact between the host stomach and adherent pathogens. Several studies have linked *MUC1* polymorphisms with susceptibility to *H. pylori*-induced disease, such as gastritis and gastric cancer (Carvalho et al., 1997; Vinall et al., 2002), suggesting a direct effect of *MUC1* polymorphisms in the development of *Helicobacter*-associated pathology. Additionally, MUC1 limits *H. pylori* infection both by steric hindrance and by acting as a releasable decoy (Linden et al., 2009; Every et al., 2008).

It has been shown that the expression of gastric mucins and trefoil factors as well as their distribution in the stomach alter in *H. pylori*-induced gastric pathologies (Lindén et al., 2002, Schmitz et al., 2009; Wang & Fang, 2003). MUC1 has been shown to be expressed early in the *H. pylori* infection process but is decreased during the metaplastic stage (Wang and Fang, 2003). MUC5AC is present in a normal stomach, but its expression is lost in gastric adenocarcinoma (Ho et al., 1995; Wang and Fang, 2003; Pinto-de-Sousa et al., 2004; Dhar et al., 2005; Roessler et al., 2005; Babu et al., 2006).

On the contrary, MUC5B, which is not expressed in a healthy human stomach, has been found in gastric adenocarcinoma biopsies (Wang and Fang, 2003; Pinto-de-Sousa et al., 2004; Roessler et al., 2005; Babu et al., 2006).

MUC6 is expressed at high levels in a normal human stomach in the mucus neck glands but is absent in the gastric epithelium altered by gastric cancer (Ho et al., 1995; Pinto-de-Sousa et al., 2004).

As described above, the intestinal mucins MUC2, MUC4 and MUC13 are not expressed in the healthy gastric mucosa but have been detected in gastric adenocarcinomas and in early events of the carcinogenesis process, such as intestinal metaplasia (Mejías-Luque et al., 2010; Reis et al., 1999; Shimamura et al., 2005; Sheng et al., 2011). Intestinal metaplasia is induced by the loss of parietal cells during *H. pylori* infection. Two distinct types of mucous metaplasia have been described: intestinal metaplasia and spasmolytic polypeptide-expressing metaplasia (SPEM). SPEM might even progresses into dysplasia (Weis et al., 2013; Wang et

al., 2000; Houghton et al., 2004). It has been suggested that intestinal metaplasia develops in the presence of pre-existing SPEM, supporting the role of SPEM as a neoplastic precursor in the carcinogenesis cascade (Yoshizawa et al., 2007; Goldenring et al., 2010).

TFF1 has been shown to be lost in 50% of gastric carcinomas (Muller and Borchard, 1993; Wong et al., 1999), while TFF2 expression has been detected in human stomach biopsies showing gastritis and atrophy but not during intestinal metaplasia and gastric carcinoma (Hu et al., 2003; Dhar et al., 2005). On the contrary, TFF3, which is not expressed in a healthy stomach, was detected in the stomach as it progressed through the intestinal metaplasia stage to gastric cancer (Taupin et al., 2001). For the aforementioned studies, mucin and trefoil factor expression was characterized by both RNA expression and immunohistochemical stainings.

The C57BL/6 mouse strain has been shown to develop gastric adenocarcinoma after long-term *H. felis* infection. This model was used to determine whether murine mucin (Muc) and trefoil factors (Tffs) expression after infection was changed in a similar fashion to the changes seen in the human gastric mucosa after *H. pylori* infection (Schmitz et al., 2009). In this study, alterations in mucin expression, including increased expression of Muc4 and Muc5b and loss of Muc5ac, occurred at 4 weeks post-infection, before the development of mucous metaplasia or gastric dysplasia (Schmitz et al., 2009). *H. felis* infection in mice is often used as an animal model to study *H. pylori*-related gastric pathologies in man. Due to the lack of several important *H. pylori* virulence factors (see below), researchers must be aware that *H. felis* might not always have similar outcomes in pathogenicity compared to *H. pylori*. Extrapolation of data obtained in a *H. felis* infection mouse model to *H. pylori* infections in humans should therefore be done with caution.

4. Virulence factors of gastric *Helicobacter* species

The virulence factors involved in the pathogenesis of a *H. pylori* infection have been tremendously studied in the past (Figure 7). The genomes of *H. felis*, *H. bizzozeronii*, *H. heilmannii*, *H. ailurogastricus* and *H. suis* have recently been published (Arnold et al., 2011; Schott et al., 2011; Smet et al., 2012; Vermoote et al., 2011; Joosten et al., 2015). The availability of these genomes will now provide platforms to identify the genes that may contribute to these pathogens virulence and carcinogenic properties as well as for further comparative analyses of genomic variability, plasticity and bacterial evolution. This chapter will focus on the *H. pylori* virulence factors involved in colonization and induction of gastric

lesions. Absence/presence of homolog genes encoding these virulence factors in the available gastric NHPH genomes will be shown as well.

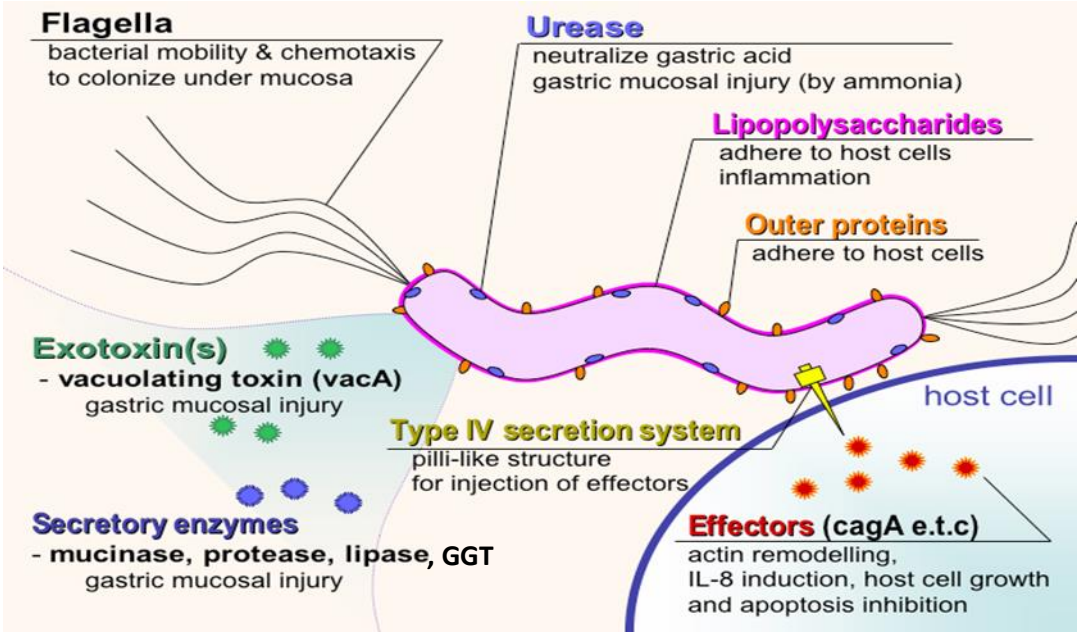

Figure 7: Virulence factors of *H. pylori* involved in gastric colonization and induction of gastric lesions. (Adapted from https://upload.wikimedia.org/wikipedia/commons/9/9a/H_pylori_virulence_factors_en.png)

4.1. Virulence factors essential for colonization of the gastric mucosa

4.1.1. pH homeostasis

For a long time, it was believed that the stomach was too hostile for persistent colonization by bacteria because of the low pH (Burne and Chen, 2000). All gastric *Helicobacter* species are able to survive the acidic environment in the stomach (Weeks et al., 2000; Pot et al., 2007). They possess a family of genes encoding the urease enzyme (Burne and Chen, 2000; Arnold et al., 2011; Schott et al., 2011; Vermoote et al., 2011; Smet et al., 2011; Figure 8). The urease enzyme consists of two subunits, UreA and UreB, and forms an essential component of *Helicobacter* acid resistance, by converting urea into ammonia and carbon dioxide. The ammonia on its turn neutralizes the hydrochloric acid of the stomach resulting in an increase of the pH in the stomach (Eaton et al., 1991; Tsuda et al., 1994; Karita et al., 1995). Downstream of the *ureAB* genes, the urease gene cluster contains a second operon, comprising *ureI* and the urease accessory *ureEFGH* genes (Akada et al., 2000). The UreEFGH proteins probably play a role in the subunit assembly and the regulation of urease activity by the incorporation of nickel (Cussac et al., 1992; Mobley et al., 1995). UreI functions as an acid-activated urea channel and controls the urea transport into the bacterial cell (Scott et al., 2000). The urease enzyme is mainly localized in the cytoplasm but is also

found on the surface of viable bacteria after autolysis of surrounding bacteria (Phadnis et al., 1996; De Reuse et al., 2005).

4.1.2 Motility and chemotaxis

For many pathogenic bacteria, flagellum-dependent motility and chemotaxis are crucial factors in the process of colonization of the host organism and establishment of a successful colonization (Moens and Vanderleyden, 1996; Ottemann et al., 1997). All gastric *Helicobacter* spp. possess the ability to move to the epithelial cell surface of the stomach and thus to a more neutral pH with the assistance of highly mobile flagellae (Salama et al., 2013; Arnold et al., 2011; Schott et al., 2011; Vermoote et al., 2011; Smet et al., 2011; Figure 7). These flagellae consist of a body, hook and flagellar filament. The filaments are composed of two flagellins A (FlaA) and B (FlaB) which are important for motility (Josenhans et al., 1995; Andrutis et al., 1997). The flagellar hook (FlgE) and flagellar cap (FliD) proteins have also been shown to be essential in flagellar structure and motility (O'Toole et al., 1994; Kim et al., 1999).

In addition to motility, several *in vivo* studies indicated that chemotaxis is also required for gastric *Helicobacter* colonization (Foynes et al., 2000; McGee et al., 2005; Terry et al., 2005). These bacteria show a directed motility towards urea, bicarbonate, cholesterol, arginine and other amino acids, but move away from hydrochloric acid. The chemotaxis receptor TlpB is required for pH taxis. *Helicobacter* bacteria lacking this receptor are defective for colonization (O'Toole et al., 1994; Kim et al., 1999). Compared to *H. pylori*, *H. heilmannii*, *H. felis*, *H. bizzozeronii* and *H. suis* possess a larger number of methyl-accepting chemotaxis proteins, allowing them to respond to a wider spectrum of environmental signals. The high metabolic versatility and the ability to react to a range of environmental signals, which differentiate these NHPH from *H. pylori*, may contribute to their zoonotic nature (Arnold et al., 2011; Schott et al., 2011; Vermoote et al., 2011; Smet et al., 2011).

4.1.3 Oxidative stress

Next to the acidic environment, gastric helicobacters have also to counteract oxidative stress, resulting from the host immune response. They all possess several key components, including catalase (KatA), superoxide dismutase (SodB) and alkyl hydroperoxide reductase (AhpC) Seyler et al., 2001; Arnold et al., 2011; Schott et al., 2011; Smet et al., 2011). The neutrophil-activating protein (NAP), present among all gastric *Helicobacter* species (Arnold et al., 2011;

Smet et al., 2011; Schot et al., 2011; Vermoote et al., 2011), has been shown to play a role in the induction of neutrophils to produce reactive oxygen radicals and combating oxidative stress resistance during *H. pylori* colonization (Olczak et al., 2003; de Bernard et al., 2010; Evans et al., 1995).

4.1.4 Outer membrane proteins

One of the very first steps in the pathogenesis of gastric infections caused by *Helicobacter* is colonization, and in particular adhesion of these bacteria to the gastric mucosa (McGuckin et al., 2011).

H. pylori is equipped with a set of ca. 64 outer membrane proteins (OMPs), whose role in the colonization process has been well studied (Alm et al., 2000; Odenbreit et al., 2009). The *H. pylori* OMPs can be divided into 5 paralogous families. The largest family comprises the Hop (for ***H****elicobacter* **o**uter membrane **p**orin, 21 members) and Hor (for **Ho**p **r**elated, 12 members) proteins. Families 2 and 3 comprise the Hof (for ***H****elicobacter* **O**MP **f**amily, 8 members) and Hom (for ***H****elicobacter* **o**uter **m**embrane, 4 members) proteins, respectively. Families 4 and 5 are composed of iron-regulated OMPs (6 members) and efflux pumps OMPs (3 members), respectively. The function of the 10 remaining OMPs is still unkown (Oleastro and Ménard, 2013; Figure 8). The *H. pylori* Hop OMPs share highly similar or identical sequences at their amino and carboxyl termini and include porins and adhesins involved in adhesion to the gastric mucosa (Alm et al., 2000; Aspholm et al., 2006b; Odenbreit, 2005; Gerhard et al., 2001). An overview of the well-studied *H. pylori* adhesins and their presence in animal-associated gastric helicobacters is given below.

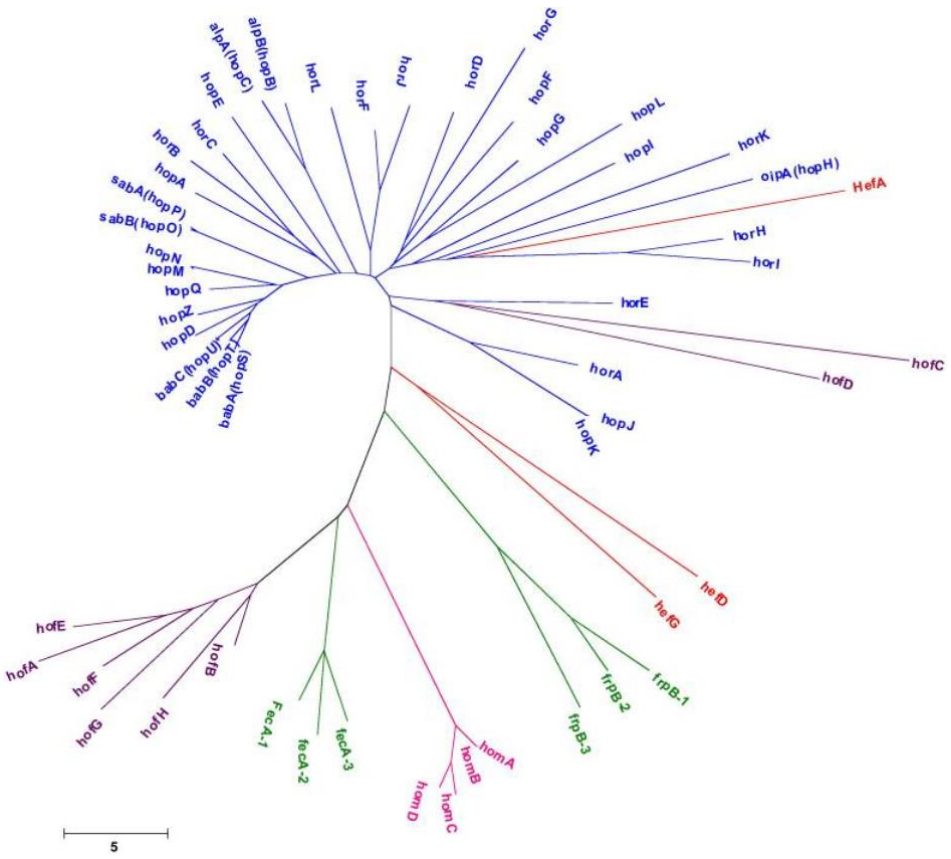

Figure 8: Phylogenetic analysis of the large set of outer membrane proteins identified in *H. pylori*. The five major families of outer membrane proteins (OMP) are represented. Family 1 (blue) comprises 21 members named Hop (21 members) and Hor (12 members) proteins. Families 2 (red) and 3 (pink) comprise the Hof (8 members) and Hom (4 members) proteins, respectively. Families 4 (green) and 5 (red) are composed of iron-regulated OMPs (6 members) and efflux pump OMPs (3 members), respectively. The 10 other non-classified OMPs are not included in the phylogenetic tree. The duplicated Hop proteins (HopJ/K and HopM/N) were not differentiated and are each visualized as one line. The branch length index is represented below the tree and highlights the differences in nucleotides. (Adapted from Alm et al., 2000 and Oleastro and Ménard, 2013).

The first and major adhesin identified in *H. pylori* is the **blood group antigen binding adhesin A** (BabA or HopS; Figure 8). BabA mediates binding of the bacterium to the human fucosylated Lewis b blood group antigen (Le^b) and related terminal fucose residues found on blood group O (H antigens), A and B antigens which are expressed on MUC1 and MUC5AC and gastric epithelial cells in the human stomach. Le^b is the dominant antigen present in the gastric mucosa (Boren et al., 1993; Ilver et al.,1998). The BabA adhesin has two closely related paralogs, BabB (HopT) and BabC (HopU), whose function has not yet been determined. The genes encoding these Bab proteins present extensive 5' and 3' homology, suggesting that the middle variable region of BabA most likely encodes the adhesin function (Pride et al., 2002). The expression level of BabA can be regulated by gene conversion, a

process by which one DNA sequence e.g. loci A replaces a homologous sequence e. g. loci B by recombination such that the sequences become identical after the conversion event. Gene conversion occurs most likely by RecA-dependent intragenomic recombination between complementary loci leading to chimeric *babB/A* and *babA/B* genes (Solnick et al., 2004; Matteo et al., 2011).

Another mechanism involved in regulation of BabA expression at transcriptional level is phase variation through slipped strand mispairing (SSM) based on the number of Cytidine-Thymidine (CT) dinucleotide repeats in the 5' region of the *babA* gene. These CT repeats are probably the result of chromosomal translocation events (Ilver et al., 1998; Solnick et al., 2004).

The second most well characterized adhesin of *H. pylori* is the **sialic acid-binding adhesin A** (SabA or HopP; Figure 8) and binds to sialylated carbohydrate structures, such as sialyl-dimeric lewis x (sLe^x), which are upregulated as part of complex gangliosides in inflamed gastric tissue (Mahdavi et al., 2002). SabA was postulated to contribute to the chronic persistence of the infection (Aspholm et al., 2006a; Mahdavi et al., 2002). SabA can also mediate the adherence of *H. pylori* to laminin (Walz et al., 2005). SabA has a closely related paralog SabB (HopO). The 5' and 3' ends of the *sabA* gene share the highest nucleotide identity with *sabB* and an additional OMP gene, *hopQ*. The OMPs encoded by these latter genes may also be involved in *H. pylori* adherence (Loh et al., 2008; Saunders et al., 2005). Gene conversion, resulting from intragenomic recombination between *sabA* and *sabB* and at a lower rate with *hopQ*, is involved in the regulation of the amount of SabA on the bacterial surface and adherence to gastric tissue (Yamaoka et al., 2006). The *sabA* gene can also be regulated at transcriptional level. Dinucleotide CT repeats present in the 5'coding region of *sabA* regulate its expression by phase variation through SSM (Yamaoka et al., 2006) and the *sabA* promoter modulates its transcriptional activity through a variable homopolymeric thymidine tract (Kao et al., 2012). The on/off switch of SabA expression suggests that SabA expression can rapidly respond to the changing conditions in the stomach (Yamaoka et al., 2006).

Two highly homologous OMPs, the **adherence-associated lipoproteins A and B** (AlpA (HopC) and AlpB (HopB); Figure 8), are also involved in *H. pylori* adherence to human gastric tissue sections (Odenbreit et al., 1996; Odenbreit et al., 1999; Alm et al., 2000; Alm et al., 1999). Both lipoproteins contribute to host laminin binding and influence gastric inflammation (Bernarde et al., 2010; Baik et al., 2004; Odenbreit et al., 1997).

The **outer inflammatory protein A** (OipA or HopH; Figure 8), encoded by the *oipA* gene, was initially identified as a pro-inflammatory response-inducing protein (Yamaoka et al., 2000). It has been shown that OipA is involved in bacterial adherence to gastric epithelial cells *in vitro* and is strongly associated with increased interleukin (IL)-8 secretion of epithelial cells in a Type 4 secretion system (T4SS)-independent manner (Dossumbekova et al., 2006; Yamaoka et al., 2000; Yamaoka et al., 2002). The *oipA* gene does not have highly similar paralogs and is quite distant from other *hop* genes. OipA expression is regulated by phase variation within a CT dinucleotide repeat motif located in the 5' coding region of the *hopH* gene. The "on" status of this adhesin has been associated with more severe gastric diseases, *H. pylori* density and severe neutrophil infiltration (Franco et al., 2008; Saunders et al., 1998).

The **HopZ** (Omp1; Figure 8) OMP has been described on the surface of *H. pylori* bacteria (Peck et al., 1999). A *hopZ*-knockout mutant strain showed significantly reduced binding capacity to the gastric epithelial AGS cell line, compared to the corresponding wild-type strain, suggesting the role of HopZ in adherence of *H. pylori* (Yamaoka et al., 2002). The expression of the *hopZ* gene is regulated by SSM within CT dinucleotide repeats of different length localized at the 5' end of the coding region. The *hopZ* "off" status frequently switches to "on" status during infection (Kennemann et al., 2012).

The smallest family of OMP is the Hom family constituted of four members. The most studied gene is *homB* encoding the **HomB** protein (Figure 8) expressed in the *H. pylori* outer membrane (Oleastro et al., 2008). This protein is associated with IL-8 secretion *in vitro* and contributes to bacterial adherence (Oleastro et al., 2008). The *homB* gene has a closely related paralog, *homA*. Expression of both loci can be regulated by gene conversion or phase variation (Oleastro et al., 2009).

HorB, a member of the Hor family (Figure 8), has also been suggested to be involved in gastric epithelial cell adhesion. Disruption of the *horB* gene reduced *H. pylori* adhesion to gastric epithelial cells (Snelling et al., 2007).

The animal-associated gastric *Helicobacter* spp. also harbour a large set of ca. 55-60 OMPs which is in agreement with the 64 well annotated OMP genes described in *H. pylori* (Joosten et al., 2015; Alm et al 2000). Comparative genomics between the *H. pylori* and the available NHPH genomes revealed that all canine, feline and porcine helicobacters lack the *H. pylori* Hop and Hom adhesins described above (Joosten et al., 2015). Among *H. heilmannii*, *H. ailurogastricus*, *H. felis*, *H. bizzozeronii* and *H. suis*, only a few homologs encoding members of the *H. pylori* Hop and Hor family, including HorB (probably functioning as a porin) are

present (Joosten et al., 2015). On the contrary, *H. acinonychis* and *H. cetorum*, the 2 most closest relatives of *H. pylori*, harbor a few homologs of known *H. pylori* Hop adhesins, such as SabB, AlpA/B and OipA (Smet et al., 2011, Arnold et al., 2011, Vermoote et al., 2011, Schott et al., 2011; Joosten et al., 2015). Which OMPs are involved in binding to the gastric mucosa by *H. heilmannii*, *H. ailurogastricus*, *H. felis*, *H. bizzozeronii* and *H. suis* is so far unknown. Interestingly, homologs of all members of the *H. pylori* Hof family are present and well conserved among all gastric helicobacters, with the exception of *H. mustelae* (Figure 8, Smet et al., 2014). In contrast to *H. pylori*, the genes encoding the Hof OMPs and present in the *H. heilmannii*, *H. ailurogastricus*, *H. felis*, *H. bizzozeronii* and *H. suis* genomes are located in a large locus. In *H. pylori*, *H. cetorum* and *H. acynonichis*, the *hof* genes are scattered across their genomes. The role of such *hof* gene locus in gastric colonization has not yet been investigated (Joosten et al., 2015).

4.2 Virulence factors involved in the induction of gastric lesions

4.2.1 Vacuolating cytotoxin

The vacuolating cytotoxin (VacA; Figure 7) is one of the major virulence factors of *H. pylori* and is one of the most extensively studied toxins produced by *H. pylori* (Leunk et al., 1988; Cover et al., 1992). The VacA toxin binds to host cells after secretion through a type V autotransport secretion system. The induction of vacuolation in host cells, caused by VacA, has been observed in primary gastric epithelial cells (Garner et al., 1996; Smoot et al., 1996; de Bernard et al., 1997; Palframan et al., 2012).

In addition to the induction of vacuolation, VacA exerts a variety of other effects on target cells, including disruption of mitochondrial functions (Kuck et al., 2001), stimulation of apoptosis and blockade of T-cell proliferation (Cover and Blanke, 2005). VacA is also important for colonization of *H. pylori in vivo* (Salama et al., 2001). Infection with *H. pylori* strains containing the toxigenic allelic s1 form of VacA is associated with an increased risk of peptic ulceration and gastric cancer (Atherton et al., 1995;Gerhard et al., 1999; Miehlke et al., 2000; Louw et al., 2001). VacA is absent in *H. heilmannii*, *H. ailurogastricus*, *H. felis*, *H. bizzozeronii*, *H. suis* and *H. acynonichis.* Homologs of *H. pylori* VacA have only been found in *H. cetorum* (Joosten et al., 2015; Kersulyte et al., 2013). Additionally, *H. pylori* contains 3 genes annotated as putative *vacA* paralogs because the C-terminal autotransporter domains of their encoded proteins show approximately 30% identity to that of VacA. These 3 VacA-like proteins each enhance the capacity of *H. pylori* to colonize the stomach (Radin et al., 2013).

Such *vacA* paralogs have also been described among the animal-associated gastric *Helicobacter* spp. (Joosten et al., 2015).

4.2.2 Cytotoxin-associated gene pathogenicity island

The Cytotoxin-associated gene pathogenicity island (*Cag*PAI; Figure 7) consists of about 30 genes depending on the clinical strain (Akopyants et al., 1998), in which the cytotoxin-associated gene A (CagA) is a marker for the *H. pylori Cag*PAI. CagA is recognized as another key virulence factor secreted by *H. pylori* (Cover et al., 1990). *Cag* PAI encodes a T4SS which mediates the injection of cytotoxin-associated effector protein A (CagA) into epithelial cells (Odenbreit et al., 2000; Yamazaki et al., 2003). The CagA is associated with the development of peptic ulcer disease, gastric cancer, and other severe *H. pylori*-associated pathologies (Bourzac et al., 2005; Salama et al., 2013).The roles of many of the proteins encoded within the *Cag* PAI are not known. The CagI has been considered as an accessory component of the CagA secretion system not translocated into host cells and is located in the bacterial inner and outer membrane (Wang et al., 2012). Besides CagI, there are other Cag-PAI genes required for CagA delivery or localization.

After translocation into the host cell, CagA affects cell shape, increases cell motility, abrogates junctional activity, and promotes an epithelial to mesenchymal transition-like phenotype (Buti et al., 2011). The inhibition effect of CagA on apoptosis in human epithelial cells has been described in some reports. It has also been shown that CagA increases apoptosis of human gastric adenocarcinoma cells (AGS) (Tsutsumi et al., 2003). Human infections with CagA-positive *H. pylori* strains mainly show overexpression of pro-apoptotic proteins. Interestingly, this *Cag* PAI is absent among all known animal-associated gastric *Helicobacter* genomes.

4.2.3 Gamma-glutamyltranspeptidase

The gamma-glutamyltranspeptidase (GGT) is an important virulence factor, and is well conserved in the genus *Helicobacter* (Smet et al., 2011; Vermoote et al., 2011; Arnold et al., 2011; Schott et al., 2011; Rossi et al., 2012; Haesebrouck et al., 2009; Wachino et al., 2010; Figure 7). Membrane-assocated *H. pylori* GGT activity plays an important role in the metabolism of gluthatione (Orlowski and Meister, 1970), a free thiol maintaining an optimal intracellular redox environment (Circu et al., 2010). The *Helicobacter* GGT is very efficient in using both glutamine and gluthatione from epithelial cells as a source of glutamate

(Shibayama et al., 2003; Flahou et al., 2011). As glutamine and gluthatione are important nutrients to maintain a healthy gastric tissue, their depletion by the GGT probably plays a role in damaging of gastric epithelial cells. Previous reports have shown that the *H. pylori* GGT is involved in induction of host cell apoptosis via a mitochondria-mediated pathway (Shibayama et al., 2001; Shibayama et al., 2003; Kim et al., 2007). Flahou and colleagues (2011) showed that the *H. suis* GGT catalyses the degradation of extracellular glutathione. The degradation products cause a cell-independent extracellular generation of H_2O_2, leading to lipid peroxidation and necrosis. During this process, inflammation-promoting molecules are released from gastric epithelial cells (Flahou et al., 2011). Additionally, the *Helicobacter* GGT has also been shown to inhibit T cell proliferation, thereby indicating its role in evasion of host immune response (Schmees et al., 2007; Zhang et al., 2015). The importance of *H. pylori* GGT in pathogenesis is further supported by studies demonstrating that *H. pylori* GGT upregulates COX-2 and EGF-related peptide expression in human gastric cells (Busiello et al., 2004; Gong et al., 2010).

4.2.4 Other virulence factors

Besides VacA, cagPAI and GGT, other *H. pylori* virulence factors have also been associated with gastric disease, including NapA, PutA, PutP, AnsB, FldA, PgbA-B, HtrA, PrtC and IceA. Homologs encoding these virulence factors have been detected in the available *H. heilmannii*, *H. felis*, *H. bizzozeronnii*, *H. ailurogastricus*, *H. suis*, *H. acinonychis* and *H. cetorum* genomes (Joosten et al., 2015; Smet et al., 2011; Schott et al., 2011; Arnold et al., 2011; Vermoote et al., 2011; Kersulyte et al., 2013). NapA has been identified as a immunomodulator. The *H. pylori* flavodoxin protein (FldA) is an electron acceptor of the pyruvate oxidoreductase enzyme complex, which has been associated with MALT lymphoma (Chang et al., 1999).

The ulcer-associated protein restriction endonuclease (IceA) has been shown to be induced by contact of the bacterium with the gastric epithelium (Smet et al., 2011). IceA is present in most gastric helicobacters and has been identified as a virulence factor associated with peptic ulcer disease (Peek et al., 1998; Donahue et al., 2000). There are two distinct genotypes in the *iceA* gene, iceA1 and iceA2. Only *iceA1* RNA is induced following adherence (Peek et al., 2000). The *H. heilmannii* strains tested so far all contained an intact *iceA1* homolog (Joosten et al., 2015). Another virulence factor, high temperature requirement A (HtrA), is involved in the cleavage of E-cadherin and thus in the disruption of gastric epithelial barrier junctions,

facilitating intercellular entry of *H. pylori* (Hoy et al., 2010; Lower et al., 2011). Recently it has been demonstrated that *H. pylori* induces ectodomain cleavage of E-cadherin in polarized cells (Weydig et al, 2007). AnsB and L-asparaginase (DcuA) are involved in cell-cycle inhibition. The PgbA-B proteins play a role in plasminogen binding while collagenase (PrtC) is an enzyme which in charge of the degradation of collagen.

Although a substantial number of genes encoding putative virulence factors have been described to be present in the NHPH genomes in general and in the *H. heilmannii* genome in particular, for the majority of these, their possible role in pathogenesis still remains unclear.

5. Innate immune response upon *Helicobacter* adherence to the gastric mucosa

Helicobacter spp. generally cause a gastric life-long infection and the induced host immune response does not result in eradication of the infection (Flahou et al., 2012). In contrast to the scarce literature about the host immune responses against NHPH, several studies describing the host immune response (both the innate and adaptive host responses) against *H. pylori* are available. This section therefore particularly focuses on *H. pylori*. More specifically, it intends to give an overview of the innate immune responses upon adherence of *H. pylori* to the gastric mucosa. Where possible, information about NHPH-induced innate responses is introduced as well.

During *H. pylori* infection, most of the bacteria remain within the gastric mucus layer of the human stomach, while only 1% interacts directly with gastric epithelial cells (Dubois & Boren, 2007). Adhesion of *H. pylori* to gastric epithelial cells is an essential step in the induction of an immune response. The initiation of innate immune responses to bacterial pathogens is dependent on the recognition of microbial structures or products by host pattern recognition receptors (PRRs). PRRs can be located either on cytoplasmic and endosomal membranes or in the cytosol of host cells (Akira et al., 2006). The surface-expressed PRRs, Toll-like receptors (TLRs), play an important role in mucosal host defense against *H. pylori*. Although there are ten known human TLRs, only TLR2, -4, -5 and -9 have been detected on gastric epithelial cells in the human stomach (Schmausser et al., 2004) and thus may be important for the recognition of *H. pylori in vivo*. TLR2 responds to stimulation with a wide variety of microbial products, while TLR4 is rather specific for Gram-negative lipopolysaccharide (LPS). Nevertheless, some forms of Gram-negative LPS, including the *H. pylori*-derived LPS,

have a weak affinity for this receptor and mainly act via TLR2 (Schmith et al., 2003; Figure 9). Activation of the innate immunity via LPS-induced TLR2 has also been described for the canine/feline *H. felis* (Mandell et al., 2004). Despite the low affinity of *Helicobacter* LPS to TLR4, there is evidence that this receptor may play a partial role in initiating dendritic cells (DCs) to produce cytokines in response to *H. pylori* (Rad et al., 2009; Figure 9).

Another *H. pylori* component involved in TLR2 signalling is the neutrophil-activating protein (Hp-NAP). Hp-NAP is able to induce the production of IL-12 and IL-23 by human neutrophils and monocytes, thereby facilitating the differentiation of native T-helper (Th) cells into the Th1 phenotype (D'Elios et al., 2007; Figure 9). Homologs of the Hp-NAP coding gene, *napA*, are also present in the NHPH genomes (Arnold et al., 2011; Vermoote et al., 2011; Schott et al., 2011; Smet et al., 2013), but their role in the activation of the innate immunity via TLR signalling remains to be further elucidated.

Bacterial flagellin is specifically recognized by TLR5 (Hayashi et al., 2001). Given the fact that *H. pylori* expresses 4 to 6 polar, sheated flagella, it seemed reasonable to assume that TLR5 is involved in *H. pylori* flagellin recognition (Eaton et al., 1996). However, it has been shown that the *H. pylori* flagellin molecules cannot be sensed by TLR5, because these molecules lack the conserved amino acids required for full TLR5 agonist activity (Andersen-Nissen et al., 2005; Figure 9).

In addition to TLRs, host cells may also express cytosolic pathogen recognition molecules, such as the nucleotide-binding oligomerization domain (NOD) proteins. These molecules have been shown to play an important role in host defense against bacterial pathogens, including *H. pylori* (Ferrero, 2005). *H. pylori* triggers NOD1 signalling via its T4SS. More specifically, *H. pylori* activates NOD1 by transfer of its peptidoglycan into the cytoplasm of epithelial cells via a *cag*PAI-dependent mechanism (Viala et al., 2004; Figure 9).

Both *H. pylori*-mediated TLR2 and NOD1 signalling result in the induction of NF-κB and a subsequent production of pro-inflammatory cytokines, including IL-8 (Viala et al., 2004). Production of IL-8 in gastric epithelial cells can also be mediated in a TLR-/NOD1-independent manner by *H. pylori* OMPs (Oleastro & Menard, 2013; Figure 9). OipA or HopH regulates IL-8 secretion through PI3/Akt and this regulation is dependent on FoxO1/3a inactivation. By using the BabA and SabA adhesins, *H. pylori* can bind via carbohydrate structures to gastric mucins, such as MUC1. On its turn, this mucin is able to induce IL-8 in gastric epithelial cells (Sheng et al., 2013; Figure 9). Also other *H. pylori* OMPs involved in attachment to gastric epithelial cells, including AlpA/AlpB and HomB, have been shown to

trigger gastric epithelial IL-8 secretion upon adherence (Oleastro & Menard, 2013; Ishijima et al., 2011; Figure 9).

The production of pro-inflammatory cytokines by gastric epithelial cells after *H. pylori* infection facilitates a second stage in the innate immune response which involves the recruitement of innate immune cells (neutrophils, macrophages and DCs) to the site of infection. Phagocytosis mediated by macrophages and neutrophils is an important anti-bacterial innate defense mechanism. *H. pylori* partially evades phagocyte-mediated killing by surviving inside phagosomes. This may provide a protected intracellular niche contributing to the persistence of infection (Allen et al., 2000; Allen et al., 2001). Additionally, *H. pylori* induces the extracellular release of ROS by phagocytes, but the bacterium is capable of surviving this response through its catalase and superoxide dismutase activity (Ramarao et al., 2000).

In response to *H. pylori* antigens, DCs and macrophages further amplify the inflammatory response by the production of cytokines, including IL-1β, TNF-α and IL-6. This leads to recruitment, differentiation and activation of $CD4^+$ Th cells, which organize the adaptive immune response (Wilson & Crabtree, 2007; Figure 9).

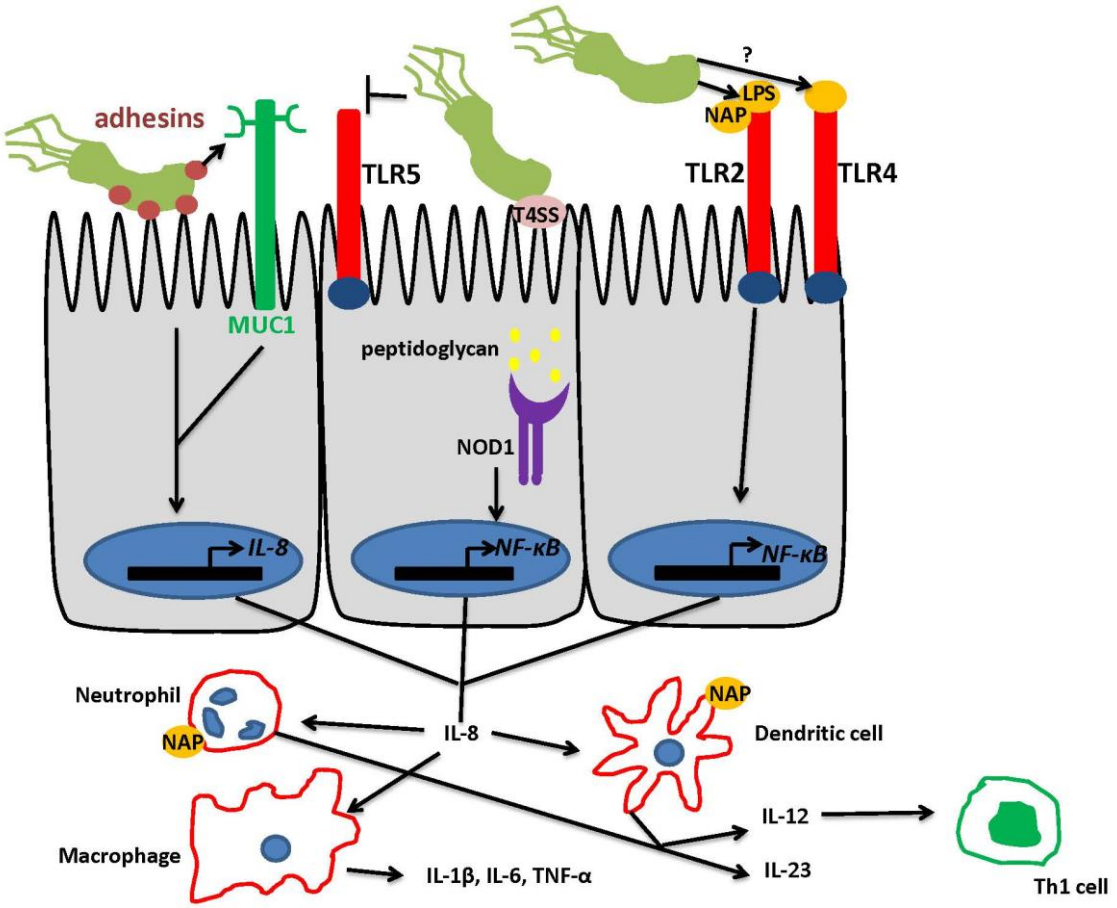

Figure 9: Schematic representation of the innate immune response upon *H. pylori* adherence via pathogen-recognition molecule (PRM) and outer membrane protein (OMP) signalling. *H. pylori* pathogen-associated molecular patterns (PAMPs) (e.g. LPS, NAP) are detected by PRMs expressed by epithelial cells, which results in the activation of NF-κB and the production of pro-inflammatory cytokines, such as IL-8. Additionally, *H. pylori* adhesins (including AlpA/B, HomB, BabA and SabA) can initiate IL-8 production upon adherence. The pro-inflammatory cytokine IL-8 recruits macrophages, neutrophils and dendritic cells to the site of infection, which can be further activated by PAMPs that pass through the epithelial cell barrier. These activated immune cells subsequently produce cytokines that facilitate the recruitment of T-helper cells, resulting in the progression of inflammation and the development of an adaptive immune response.

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SCIENTIFIC AIMS OF THE STUDY

Gastric disease in humans has not only been associated with the well-studied pathogen *Helicobacter pylori,* but also with other *Helicobacter* spp. naturally colonizing the stomach of domestic animals such as *H. heilmannii*. This zoonotic gastric NHPH species is highly prevalent in both cats and dogs. It has been associated with gastritis, gastric and duodenal ulcers and low grade MALT lymphoma in humans. Living in close contact with cats and dogs has been identified as a significant risk factor for these infections in humans. Information on how *H. heilmannii* interacts with its host still remains poor. The very fastidious nature of this bacterium has hampered the progress of *H. heilmannii*-related research. *H. heilmannii* was successfully isolated *in vitro* in 2011 opening new doors to investigate the role of this pathogen in human gastric pathology.

The **ultimate goal** of this thesis was to identify the bacterial and host factors involved in colonization of the gastric mucosa by *H. heilmannii*.

The human gastric mucosa is covered by a layer of continuously secreted mucus that washes away trapped particles. It is the first barrier *Helicobacter* encounters and consists of heavily glycosylated secreted mucins. The gastric epithelium underneath the mucus layer consists of various cell types. To obtain better insights into the interaction of *H. heilmannii* with the human gastric mucosa, experimental infection studies with this bacterium are essential. Rodents have been shown to be useful models for the study of *Helicobacter*-related human gastric disease. The **first** specific **aim** of this thesis was to investigate the distribution and expression pattern of mucins in the stomach of mice during a one year infection with *H. heilmannii* in which gastric disease progressed in severity.

Adherence to the gastric mucosa is widely assumed to play not only an important role in the initial colonization but also in the long-term persistence of *Helicobacter* in the stomach which may last for decades or even an entire lifetime. It is well-known that the vast majority of *Helicobacter* bacteria remains in the mucus layer by adherence to mucins, while a small percentage binds to the epithelial cell surfaces underneath the mucus layer. Bacterial OMPs are directly involved in the interaction of pathogenic bacteria with their host. For *H. heilmannii*, however, nothing is known about the virulence factors that play a role in binding of this bacterium to the gastric mucosa. Thus, the **second specific aim** of this thesis was to

characterize the *H. heilmannii* OMPs involved in this process. Additionally, their role in gastric mucin expression during *H. heilmannii* colonization was investigated as well.

EXPERIMENTAL STUDIES

1. **Gastric de novo Muc13 expression and spasmolytic polypeptide-expressing metaplasia during *Helicobacter heilmannii* infection**
2. **The *Helicobacter heilmannii hofE* and *hofF* genes are essential for colonization of the gastric mucosa and are involved in IL-1β-induced gastric MUC13 expression**

Gastric *de novo* Muc13 expression and spasmolytic polypeptide-expressing metaplasia during *Helicobacter heilmannii* infection

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Abstract

Helicobacter heilmannii is a zoonotic bacterium that has been associated with gastric disease in humans. In this study, mRNA expression of mucins was analyzed at several time-points in the stomach of BALB/c mice during a one year infection with this bacterium in which gastric disease progressed in severity. Markers for acid production by parietal cells and mucous metaplasia were also examined. In the first 9 weeks post-infection, mRNA expression of Muc6 was clearly upregulated in both the antrum and fundus of the stomach of *H. heilmannii*-infected mice. Interestingly, Muc13 was upregulated already at 1 day post-infection in the fundus of the stomach. Its expression level remained high in the stomach over the course of the infection. This mucin is, however, not expressed in a healthy stomach and a high expression of this mucin has so far only been described in gastric cancer. In the later stages of infection, mRNA expression of H^+/K^+-ATPase α/β and KCNQ1 decreased whereas the expression of Muc4, Tff2, Dmbt1 and PigR increased starting at 16 weeks post-infection onwards suggesting the existence of spasmolytic polypeptide-expressing metaplasia in the fundus of the stomach. Mucous metaplasia present in the mucosa surrounding low grade mucosa associated lymphoid tissue lymphoma-like lesions was also histologically confirmed. Our findings indicate that *H. heilmannii* infection causes severe gastric pathologies, alterations in the expression pattern of gastric mucins, such as Muc6 and Muc13, as well as a disruption in the gastric homeostasis by inducing loss of parietal cells resulting in the development of mucous metaplasia.

Introduction

Helicobacter pylori is the major predisposing factor for the development of chronic active gastritis, peptic ulcers and gastric adenocarcinomas in humans, and approximately 15% of the infected individuals are estimated to develop such symptoms. This pathogen can be detected attached to gastric epithelial cells but is found mainly within the mucus layer and is able to bind to highly glycosylated mucins. MUC1, MUC5AC and MUC6 are the major mucins covering the healthy gastric mucosa. The membrane-associated MUC1 and secreted MUC5AC are expressed at the surface epithelium, whereas MUC6 is secreted by glandular cells (Linden et al., 2002; McGuckin et al., 2011; Linden et al., 2009; Taupin and Podolsky, 2003).

H. pylori infection causes alterations in the expression pattern, glycosylation and distribution of gastric mucins as well as a disruption in the gastric homeostasis by inducing loss of parietal cells (Skoog et al., 2012; Weis et al., 2013). The loss of parietal cells can lead to two distinct types of mucous metaplasia: intestinal metaplasia and SPEM. It has been suggested that intestinal metaplasia develops in the presence of pre-existing SPEM, supporting the role of SPEM as a neoplastic precursor in the carcinogenesis cascade (Weis et al., 2013). The intestinal mucins MUC2, MUC4 and MUC13 are not expressed in the healthy gastric mucosa but have been detected in gastric adenocarcinomas and during stages of mucous metaplasia (Mejias-Luque et al., 2010; Reis et al., 1999; Shimamura et al., 2005).

Besides *H. pylori*, other spiral-shaped NHPH, such as *H. suis* in pigs and *H. heilmannii* (sensu stricto), *H. felis*, *H. bizzozeronii* and *H. salomonis* in cats and dogs, have been associated with gastric disease in humans (Haesebrouck et al., 2009; Smet et al., 2012). *H. heilmannii* is highly prevalent in healthy cats and dogs as well as in animals with chronic gastritis (Haesebrouck et al., 2009). In humans, this *Helicobacter* species has been associated with gastritis, gastric and duodenal ulcers and low grade MALT lymphoma. It has been detected in 8–19% of gastric biopsies with histological evidence of NHPH infection (Haesebrouck et al., 2009; Smet et al., 2012; O'Rourke et al., 2004a). Living in close contact with cats and dogs has been identified as a significant risk factor for these infections in humans (Haesebrouck et al., 2009). Since this species has only recently been isolated and cultured *in vitro*, information on how *H. heilmannii* interacts with the human stomach and causes disease still remains poor. Comparative genomic analyses showed that although the *H. heilmannii* genome contains several genes encoding homologues of known *H. pylori* virulence factors, it lacks a Cag pathogenicity island (CagPAI) as well as genes encoding a vacuolating cytotoxin VacA and

several outer membrane proteins involved in binding of *H. pylori* to the gastric mucosa, such as BabA/B, SabA, AlpA/B, OipA, HopZ, HopQ and HomB (Smet et al., 2012). Thus, factors that contribute to this pathogen's colonization properties, in particular adhesion, remain to be identified. A recent infection study in a Mongolian gerbil model for human *Helicobacter*-induced pathology showed variation in colonization capacity and virulence between different *H. heilmannii* strains isolated from the gastric mucosa of cats. These findings are most probably also relevant for infection with this bacterium in humans (Joosten et al., 2013). Unlike *H. pylori*, which is mainly observed at the surface epithelium and close to MUC1- and MUC5AC-producing cells (Linden et al., 2002; Linden et al., 2009), *H. heilmannii* is mostly found in the gastric pits as has been described for other NHPH too (Joosten et al., 2013, Flahou et al., 2010). This bacterium can be found in close association with parietal cells but is also able to bind to human mucus-secreting epithelial cells as well as to mucin samples containing highly glycosylated MUC5AC and MUC6 (unpublished data). Whether a *H. heilmannii* infection has an impact on the distribution and expression of the gastric MUC1, MUC5AC and MUC6 mucins is currently unknown. SPF inbred C57BL/6 and BALB/c mice have been shown to be useful models for the study of *Helicobacter*-related human gastric disease (Flahou et al., 2010). C57BL/6 have been described genetically as predominant Th1 responders, while BALB/c mice are mainly Th2 responders (Flahou et al., 2010). It has been shown that infection with *H. suis* induces a predominant Th17/Th2 immune response in BALB/c mice and even in C57BL/6 mice, in the absence of a Th1 response, but with a more pronounced inflammation in BALB/c mice (Flahou et al., 2012). More recently, it has been suggested that infection with *H. heilmannii* also elicits a Th2 immune response (Joosten et al., 2013). These results are in contrast to the predominant Th17/Th1 response mostly seen during *H. pylori* infection in mice (Flahou et al., 2012). Additionally, *H. pylori* can also elicit a Th2 response. Typical for *H. pylori* strains inducing a Th2 immune response is that they lack genes encoding CagPAI, VacA, BabA and SabA (Crabtree et al., 2002). Therefore, in the present study, we used Th2-prone BALB/c mice to investigate the expression levels of Muc1, Muc5ac and Muc6 in the stomach at several time-points during a one year *H. heilmannii* infection in which gastric disease progressed from gastritis to MALT lymphoma-like lesions and mucous metaplasia. Since *H. heilmannii* has been found close to parietal cells in the gastric pits, markers for acid production by parietal cells were examined. Markers for mucous metaplasia (in particular the Muc2, Muc4 and Muc13 intestinal mucins) as a result of parietal cell loss, were included as well. Infection with the mouse adapted *H. pylori* SS1, a strain that

elicits a Th2 response, was included for the purpose of comparison (Flahou et al., 2012). This latter strain, created after several gastric passages in mices, is able to colonize multiple strains of mice contrary to its parental human strain pre-mouse (PM)SS1 (Crabtree et al., 2002).

MATERIALS AND METHODS

Animals.

Six-week old SPF, female BALB/c mice were purchased from Harlan NL (Horst, The Netherlands). The animals were housed in individual filter top cages, had free access to water and food (an autoclaved commercial diet (TEKLAD 2018S, containing 18% protein; Harlan)) throughout the experiment and were monitored daily.

The *in vivo* experimental protocol was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC 2011-155; October 27, 2011).

Cultivation of *H. heilmannii* and *H. pylori* strains used for infection.

The highly virulent *H. heilmannii* strain ASB1.4, isolated from the stomach of a kitten with gastritis, was cultivated as described previously (Smet et al., 2012; Joosten et al., 2013). After incubation under microaerobic conditions (Smet et al., 2012), the bacteria were harvested and the final concentration was adjusted to 7×10^8 viable bacteria/mL.

The mouse-adapted *H. pylori* SS1 strain (Crabtree et al., 2002) was grown for 3 days on blood agar plates (Oxoid) and further cultured overnight in Brucella broth (Oxoid) under microaerobic conditions. The optical density was then adjusted to 1.5, corresponding to approximately $1x10^9$ viable bacteria/mL.

Experimental procedure.

For each time point tested, 6 animals were intragastrically inoculated 3 times at 2 days interval with 300 µL of an ASB1.4 or SS1 bacterial suspension and 3 animals with *Brucella* broth (pH 5) serving as negative control. Inoculation was performed under brief isoflurane anaesthesia (2.5%), using a feeding needle. At 1 day, 4 days, 1, 2, 3, 4, 9, 12, 16, 20, 24, 34 and 52 weeks after the first inoculation, the animals were euthanized by cervical dislocation under deep isoflurane anaesthesia (5%). The stomach and the duodenum of each mouse were resected and samples were taken for histopathological examination and quantitative real-time (RT)-PCR analysis.

Histopathology and immunohistochemistry.

A longitudinal section, starting from the end of the forestomach and comprising the antrum and the fundus of the stomach and part of the duodenum, was fixed in 10% phosphate buffered formalin and embedded in paraffin for light microscopy. From each animal, several consecutive paraffin slides of 5 µm were cut, deparaffinized and dehydrated. Heat-induced antigen retrieval (100 °C, 20 min) was then performed in citrate buffer (pH 6) and endogenous peroxidase activity and non-specific reactions were blocked by incubating the slides with 3% H_2O_2 in methanol (5 min) and 30% goat serum (30 min), respectively. A haematoxylin/eosin (H&E) staining was performed on a first slide to score the intensity of the gastritis according to the Updated Sydney System as described previously (Flahou et al., 2010), but with some modifications as shown in Figure 1. On a second slide, B lymphocytes were visualized by immunohistochemical staining using a polyclonal rabbit anti-CD20 antibody (1/100; Thermo Scientific, Fremont, USA). On a third slide, parietal cells were identified by immunohistochemical staining using a mouse monoclonal antibody against the hydrogen potassium ATPase (1/200; Abcam Ltd, Cambridge, UK).

Muc13 expression was evaluated by immunohistochemical staining (one slide for each staining) using an in-house mouse polyclonal anti-Muc13 antibody. Incubation with primary antibodies directed against CD20 and Muc13 was followed by incubation with a biotinylated goat anti-rabbit IgG antibody (1/500; DakoCytomation, Heverlee, Belgium). Incubation with primary antibody against the hydrogen potassium ATPase and Muc13 was followed by a biotinylated goat anti-mouse IgG antibody (1/200; DakoCytomation). To reduce the background caused by secondary antibody binding to endogenous mouse IgG, mouse sections were further processed with Envision + System-HRP (DAB) (DakoCytomation). After rinsing, the sections were incubated with a streptavidin-biotin-HRP complex (DakoCytomation) and the color was developed with diaminobenzidine tetrahydrochloride (DAB) and H_2O_2.

To highlight lymphoepithelial lesions, paraffin slides were stained with a monoclonal mouse anti-cytokeratin antibody (1/50; DakoCytomation) and further processed using an EnVision™+ system for use with mouse primary antibodies (DakoCytomation).

Finally, a periodic acid-Schiff (PAS)-Alcian blue staining was performed for the differential staining of glycoproteins.

DNA extraction and quantification of colonizing *Helicobacter* spp. in the stomach and duodenum.

Samples from the fundus and the antrum of the stomach and from the duodenum of each animal were harvested into 1 mL RNA later (Ambion, Austin, TE, USA) and stored at -70 °C until RNA- and DNA-extraction. Tissue samples were then homogenized (MagNALyser, Roche, Mannheim, Germany) and RNA and DNA were separated (Joosten et al., 2013). The number of colonizing *H. heilmannii* and *H. pylori* per mg gastric tissue was determined in the DNA samples using quantitative RT-PCRs specific for the detection of *H. heilmannii* and *H. pylori* (Joosten et al., 2013, Flahou et al., 2012). *Helicobacter* standards were generated as described previously (O'Rourke et al., 2004b). One µL of DNA template was suspended in a 10 µL reaction mixture consisting of 3.5 µL HPLC water, 5 µL SensiMix™ SYBR No-ROX (Bioline Reagents Ltd, UK) and 0.25 µL of both primers (TABLE S1, Integrated DNA technologies). Standards and DNA samples were run in duplicate on a CFX96™ RT-PCR System with a C1000 Thermal Cycler (Bio-Rad, Hercules CA, USA). For quantification of *H. heilmannii* and *H. pylori* DNA in the tissue samples, the Bio-Rad CFX Manager (version 1.6) software was used.

Real Time (RT)-PCR for gene expression.

The total RNA concentration in each sample was utilized for first strand cDNA synthesis using iScript™ cDNA Synthesis Kit (Bio-Rad). Quantitative RT-PCR was carried out for measuring gene expression levels of murine gastric mucins (Muc1, Muc2, Muc4, Muc5AC, Muc5B, Muc6 and Muc13), trefoil factors (Tff1 and Tff2), Dmbt1, PigR, H^+/K^+-ATPase, Kcnq1 and Ckb. The housekeeping genes *PPIa, H2afz and HPRT* were included as reference genes. Primer sequences are shown in Table S1. Reactions were performed in 10 µL volumes containing 1 µL cDNA, 0.05 µL of both primers (TABLE S1), 3.9 µL HPLC water and 5 µL SensiMix™ SYBR No-ROX. The experimental protocol for PCR reaction (40 cycles) was performed on a CFX96™ RT-PCR System with a C1000 Thermal Cycler (Bio-Rad). Control reactions without the reverse transcriptase step were implemented to exclude DNA contamination of the RNA samples. No-template-control reaction mixtures were included and all samples were run in duplicate. Results are shown as fold changes of mRNA expression in infected animals relative to mRNA expression levels in control animals. Fold changes were calculated using the ΔΔCT method (Livak and Schmittgen, 2001) with mean of Ct-values

from three uninfected mice as control. Fold changes ≥4 were accepted as up-regulation and ≤0.25 as down regulation.

Statistical analysis.

Statistical analysis was performed using SPSS Statistics 21 software (IBM) package. Gastritis scores were analyzed using the non-parametric Mann-Whitney *U* test to compare groups. Gene expression was compared between different infected groups and controls using a Bonferoni post hoc test (ANOVA). For correlation between different variables, Spearman's rho correlation coefficients were calculated. Differences were considered statistically significant at $p \leq 0.05$.

RESULTS

Induction of inflammation by and colonization capacity of *H. heilmannii* ASB1 and *H. pylori* SS1.

In none of the non-infected control animals, gastric lymphoid lesions were present. For all these animals, histomorphology was considered to be normal, with only minor inflammatory cell infiltration in the gastric mucosa. Inflammation in ASB1.4- and SS1-infected mice was characterized by mononuclear and polymorphonuclear cell infiltration in the lamina propria mucosae, the tunica submucosa or both, depending on the individual animal. At all time-points, inflammation was observed mainly in the fundus. The fundic inflammation scores of each individual animal are shown in Figure 1A. No statistically significant difference between inflammation scores for mice inoculated with ASB1.4 or SS1 at a certain time-point was demonstrated. From 24 weeks post-infection onwards, large lymphoid aggregates of mononuclear and/or polymorphonuclear cells were mainly seen in a narrow zone in the fundus near the forestomach/stomach transition zone (Figure 1B & 1C) of both *H. heilmannii*- and *H. pylori*-infected mice. In mice infected with ASB1.4 and SS1 for at least 34 weeks, B-cell containing germinal centers were seen in those large lymphoid aggregates (Figures 1F & 1G). In several mice infected with ASB1.4 and SS1 for 52 weeks, numerous lymphoepithelial MALT-lymphoma like lesions could be detected in the gastric mucosa (Figures 1H & 1I). These were most abundant in a narrow zone in the fundus near the forestomach/stomach transition zone. In all *Helicobacter*-infected mice, mild signs of inflammation were detected in the antrum of the stomach and the duodenum at 52 weeks post-infection (Figures 1D &

1E). However, inflammation could also be noted in the junction between antrum and fundus of mice infected with *H. heilmannii* for 52 weeks (data not shown).

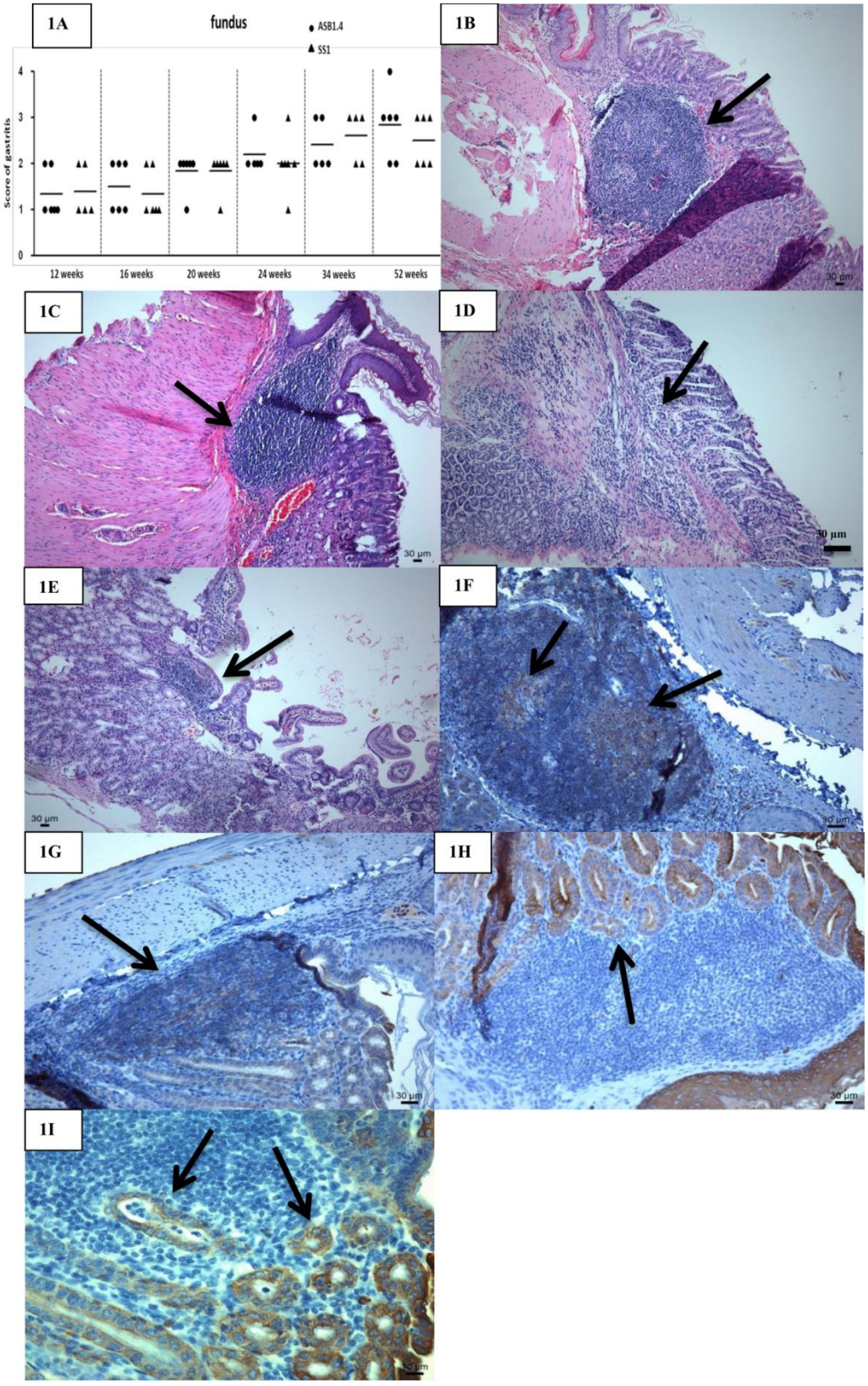

Figure 1: Gastric inflammation in *H. heilmannii*- and *H. pylori*-infected BALB/c mice.

(A) Fundic inflammation was scored on a scale of 0 to 4 (0: no infiltration with mononuclear and/or polymorphonuclear cells; 1: mild diffuse infiltration with mononuclear and/or polymorphonuclear cells or the presence of one small (50–200 cells) aggregate of inflammatory cells; 2: moderate diffuse infiltration with mononuclear and/or polymorphonuclear cells and/or the presence of 2–4 inflammatory aggregates; 3: marked diffuse infiltration with mononuclear and/or polymorphonuclear cells and/or the presence of at least five inflammatory aggregates; 4: diffuse infiltration of large regions with large aggregates of mononuclear and/or polymorphonuclear cells). Individual animals are depicted as symbols around the mean (lines). (B&E) H&E staining of the fundus, antrum and the duodenum of a *Helicobacter*-infected BALB/c mouse. A large infiltrate of mononuclear cells (arrow) at the forestomach/stomach transition zone of a mice infected with *H. heilmannii* ASB1.4 (B) and *H. pylori* SS1 (C) at 52 weeks post-infection. Bar = 30 µm. A mild lymphocytic infiltration of the lamina muscularis mucosae in the antrum (D) and duodenum (E) of a mouse infected with *H. heilmannii* ASB1.4 for 52 weeks (arrows). Bar = 30 µm. (F&G) CD20 staining of the forestomach/stomach transition zone of a mouse infected with *H. heilmannii* ASB1.4 (F) and *H. pylori* SS1 (G) at 52 weeks post-infection, showing B lymphocytes (brown) in germinal centers of lymphoid follicles (arrow). Bar = 30 µm. (H&I) Cytokeratin staining of the forestomach/stomach transition zone of a mouse infected with *H. heilmannii* ASB1.4 (H, Bar = 30 µm.) and *H. pylori* SS1 (I, Bar = 10 µm.) at 52 weeks post-infection showing numerous lymphoepithelial lesions (arrows).

Throughout the experiment, all control animals were negative for *Helicobacter* DNA in quantitative RT-PCR. At all time-points, *Helicobacter* DNA was found in both the antrum and fundus of the stomach from all infected animals. *H. pylori* and *H. heilmannii* DNA was found in the duodenum from 3 and 12 weeks post-infection onwards, respectively (Figures 2A, 2B & 2C). In general, ASB1.4 colonized the stomach of mice at a significantly higher level compared to SS1 ($P = 0.002$ for antrum at 4, 20, 24 and 34 weeks post-infection; $P = 0.004$ for antrum at 12, 16 and 52 weeks post-infection; $P=0.026$ for antrum at 9 weeks post-infection; $P = 0.009$ for fundus at 12 weeks post-infection; $P = 0.002$ for fundus at 16, 20, 24 and 34 weeks post-infection). The amount of ASB1.4 and SS1 DNA was much lower in the duodenum compared to the stomach and only at 3 weeks post-infection, a significant difference between *H. heilmannii* and *H. pylori* was seen ($P=0.015$; Figure 2C).

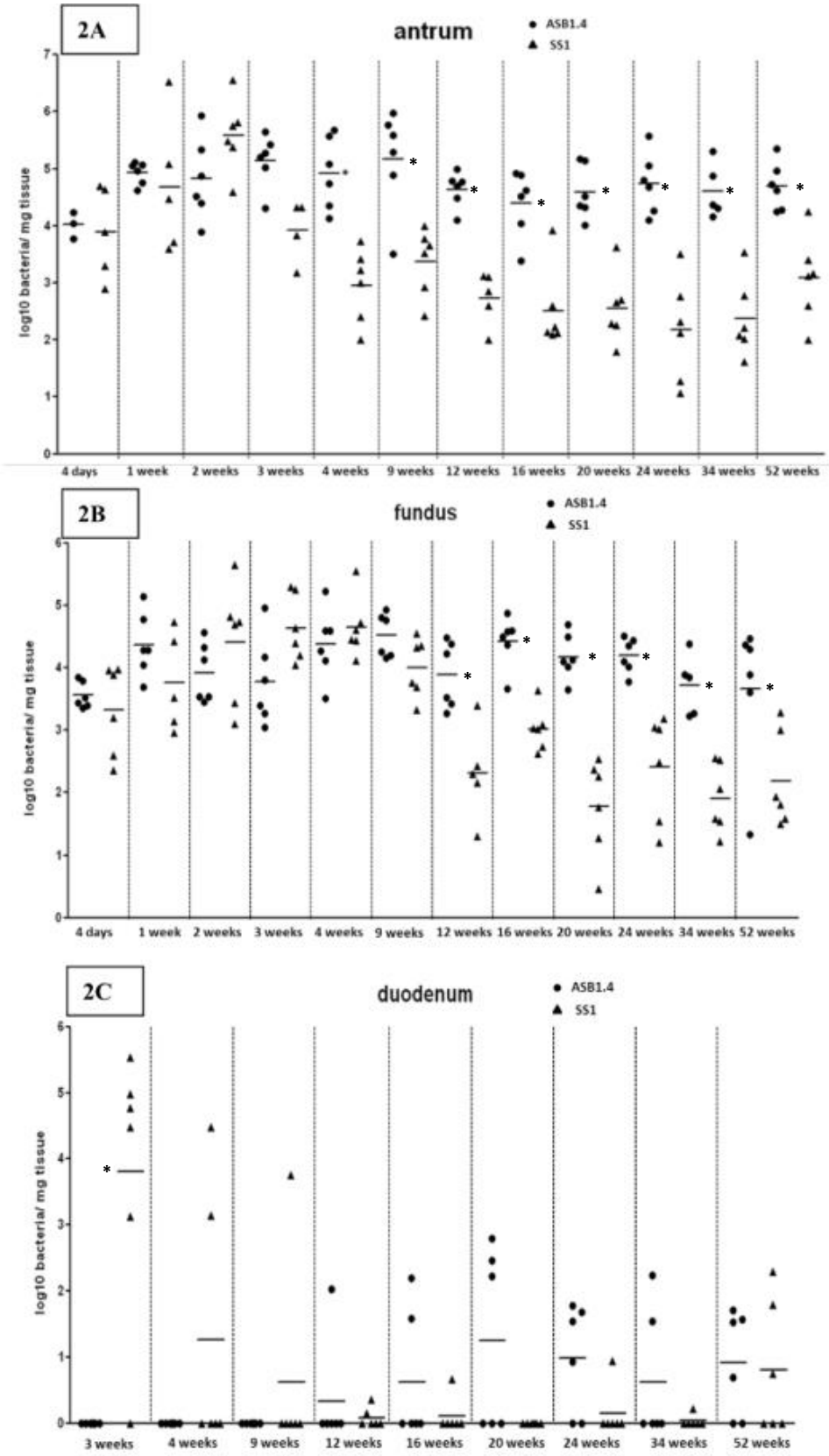

Figure 2: Colonization capacity of *H. heilmannii* ASB1.4 and *H. pylori* SS1 after experimental infection. Colonization capacity is shown as $\log_{10}$ values of bacteria per mg tissue, detected with quantitative RT-PCR in the antrum (A) and the fundus (B) of the stomach and the duodenum (C). Animals in which no *Helicobacter*

DNA could be detected in the gastro-intestinal tract were set as 0. *Helicobacter* DNA was found in the duodenum from 3 weeks post-infection onwards. Individual animals are depicted as figures around the mean (lines). Statistical significant differences between animals infected with *H. heilmannii* ASB1.4 and *H. pylori* SS1 are indicated by * (Mann-Whitney *U* test, $p < 0.05$).

Changes in Muc1, Muc5AC, Muc5B and Muc6 expression during *H. heilmannii* colonization.

No change in mRNA expression of Muc1 and Muc5AC was seen in the stomach during the whole experiment (data not shown). In the first 9 weeks post-infection, quantitative RT-PCR showed a clear upregulation in mRNA expression of Muc6 both in the antrum (ASB1.4: 7.43 ± 2.08; SS1: 6.39 ± 2.5) and fundus (ASB1.4: 5.88 ± 2.66; SS1: 6.86 ± 3.01) of *Helicobacter*-infected mice compared to the control group (Figures 3A & 3B and Supplementary figures 1A & 1B). In addition, a significant positive correlation was observed between Muc6 expression and *Helicobacter* colonization in the antrum of ASB1.4-infected mice (Figure 3C).

Also in this early stage of infection, Muc5B was abnormally expressed in the stomach of mice infected with both species (Figure 4A & 4B and Supplementary figures 1C & 1D). This mucin is normally not expressed in a healthy stomach (Schmitz et al., 2009). Compared to the control animals with mRNA expression level set to 1, the antral fold difference between Muc5B and Muc5AC was 6.02 ± 2.27 for ASB1.4- and 5.68 ± 2.81 for SS1-infected mice. The fundic fold difference between Muc5B and Muc5AC was 2.88 ± 1.63 and 3.84 ± 1.53 for ASB1.4- and SS1-infected animals, respectively.

Alterations in mucin mRNA expression were also evaluated in the duodenum. The mRNA expression level of Muc1 was significantly increased at 4 weeks post-infection in the duodenum of *H. heilmannii*-infected mice (P=0.036) and *H. pylori*-infected mice (P = 0.047) (Supplementary figure 1G). A significantly increased expression of Muc5AC was seen at 4 (P = 0.032) and 16 (P = 0.026) weeks post-infection in the duodenum of *H. heilmannii*-infected mice (Supplementary figure 1H).

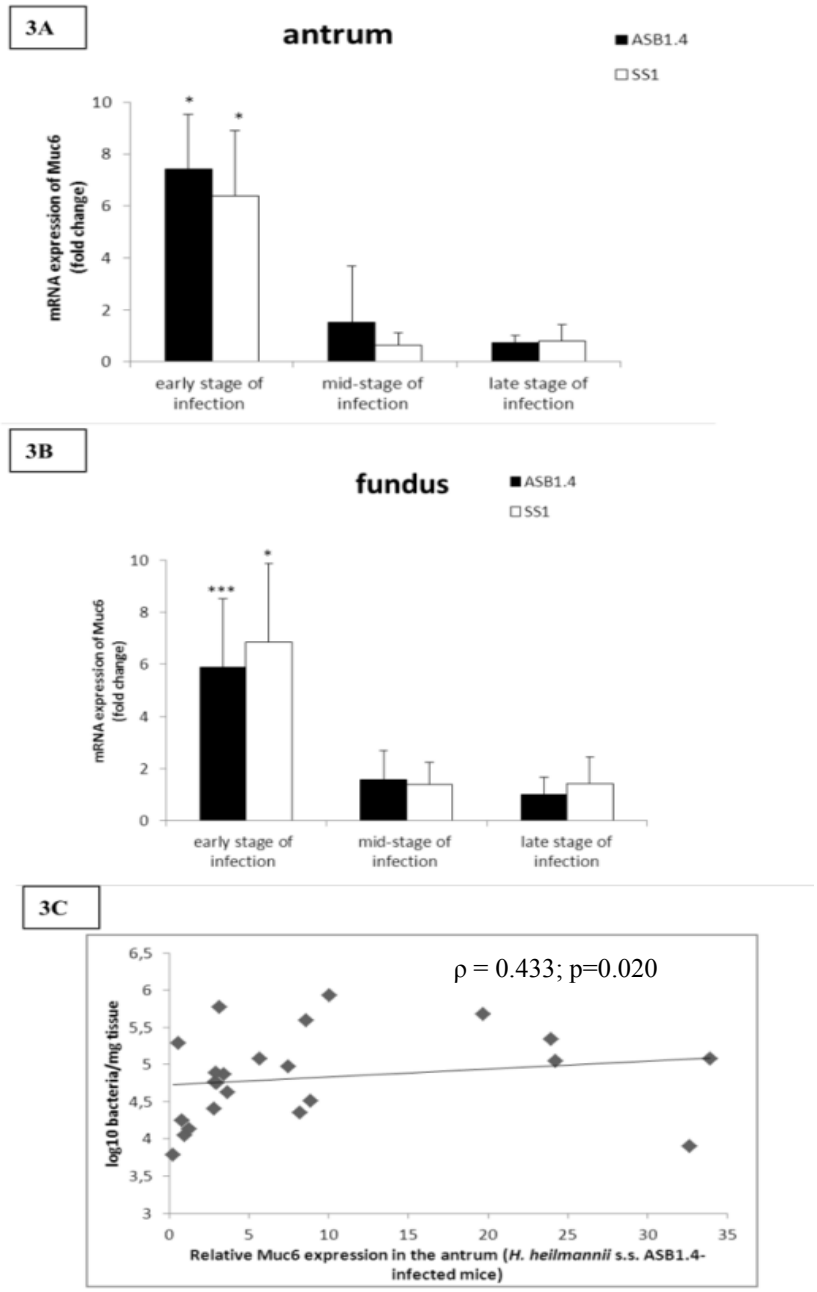

Figure 3: Muc6 expression in the stomach of *Helicobacter*-infected and control mice.

Expression level of Muc6 in the antrum (A) and fundus (B) of the stomach of ASB1.4- and SS1-infected BALB/c mice is shown. Data are presented as fold change in gene expression normalized to 3 reference genes and relative to the negative control group which is considered as 1. Data obtained from the time points 1 day, 4 days, 1, 2, 3, 4, and 9 weeks are pooled and designated as "early stage of infection". Data obtained from the time points 12, 16, 20 and 24 weeks are pooled and designated as "mid-stage of infection". Data obtained from the time points 34 and 52 weeks are pooled and designated as "late stage of infection". Data are shown as means + standard deviation. Significant differences in expression level between the infected groups and the negative control group at a certain time frame are indicated by * $p<0.05$ or *** $p<0.001$ (ANOVA). (C) Correlation analysis between Muc6 mRNA expression and the number of colonizing *Helicobacter* bacteria in the antrum of the stomach of BALB/c mice. Time-points 4 days, 1 week, 2, 4 and 9 weeks were taken into account. Correlation was measured by Spearman's Rho (ρ; $p<0.05$).

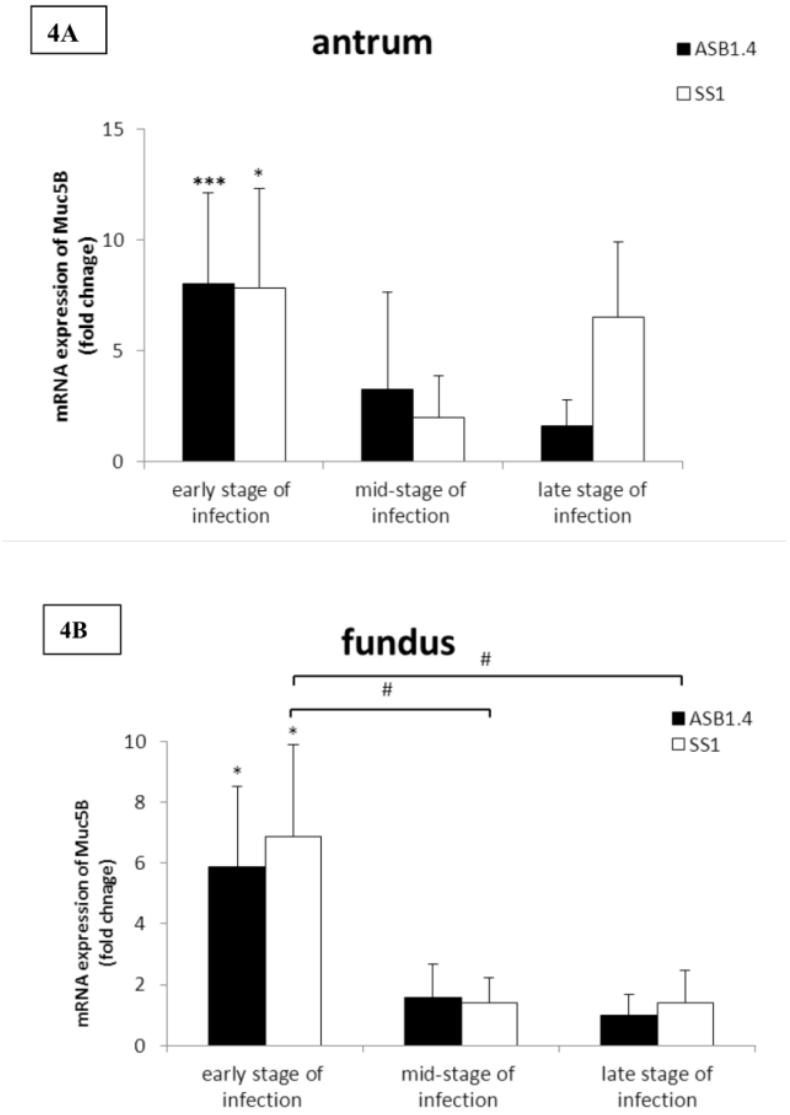

Figure 4: Muc5B expression in the stomach of *Helicobacter*-infected and control mice. Expression of Muc5B in the antrum (A) and fundus (B) of the stomach of ASB1.4- and SS1-infected BALB/c mice is shown. Data are presented as fold change in gene expression normalized to 3 reference genes and relative to the negative control group which is considered as 1. Data obtained from the time points 1 day, 4 days, 1, 2, 3, 4, and 9 weeks are pooled and designated as "early stage of infection". Data obtained from the time points 12, 16, 20 and 24 weeks are pooled and designated as "mid-stage of infection". Data obtained from the time points 34 and 52 weeks are pooled and designated as "late stage of infection". Data are shown as means + standard deviation. Significant differences in expression level between the infected groups and the negative control group at a certain time frame are indicated by * $p<0.05$ or *** $p<0.001$ (ANOVA). Significant differences in expression level between groups inoculated with *H. heilmannii* ASB1 or *H. pylori* SS1 of different time frames are indicated by # $p<0.05$ or ### $p<0.001$ (ANOVA).

***H. heilmannii* infection stimulates Muc13 expression.**

The transmembrane Muc13 is only expressed at very low levels in a healthy stomach. Under healthy conditions, this cell-surface mucin is mainly expressed in the glycocalyx of enterocytes and goblet cells in the small and large intestine particularly at the luminal surface (Sheng et al., 2011). Interestingly, mRNA expression of Muc13 was significantly increased at day 1 until 9 weeks post-infection ($P<0.001$) in the fundus of the stomach of both *H. heilmannii*- and *H. pylori*-infected mice compared to the control group (ASB1: 6.08 ± 0.84; SS1: 5.91 ± 1.21) (Figure 5B & Suplementary figure 2B). Its mRNA expression level was also high in the antrum (ASB1.4: 9.61 ± 4.08; SS1: 10.09 ± 4.38) and fundus (ASB1.4: 5.77 ± 4.33; SS1: 7.17 ± 3.67) of the stomach in the late stage of infection (Figures 5A & 5B, Supplementary figure 2A & 2B). A significant positive correlation was observed between Muc13 expression and *Helicobacter* colonization in the fundus of *H. heilmannii*- and *H. pylori*-infected mice in the first 9 weeks post-infection (Figures 5C & 5D). Although *H. heilmannii* DNA could not be found in the duodenum during the first weeks of infection, the Muc13 expression level was significantly increased here too at 1 (8.67 ± 1.89; $P<0.001$) and 2 (6.97 ±3.09; $P<0.001$) weeks post-infection (Supplementary figure 2C).

Immunohistochemical staining showed an apical membrane and cytoplasmic Muc13 staining (brown) in mucus-secreting epithelial cells of the stomach of *Helicobacter* infected mice but not in the non-infected controls (Figure 5E & 5F).

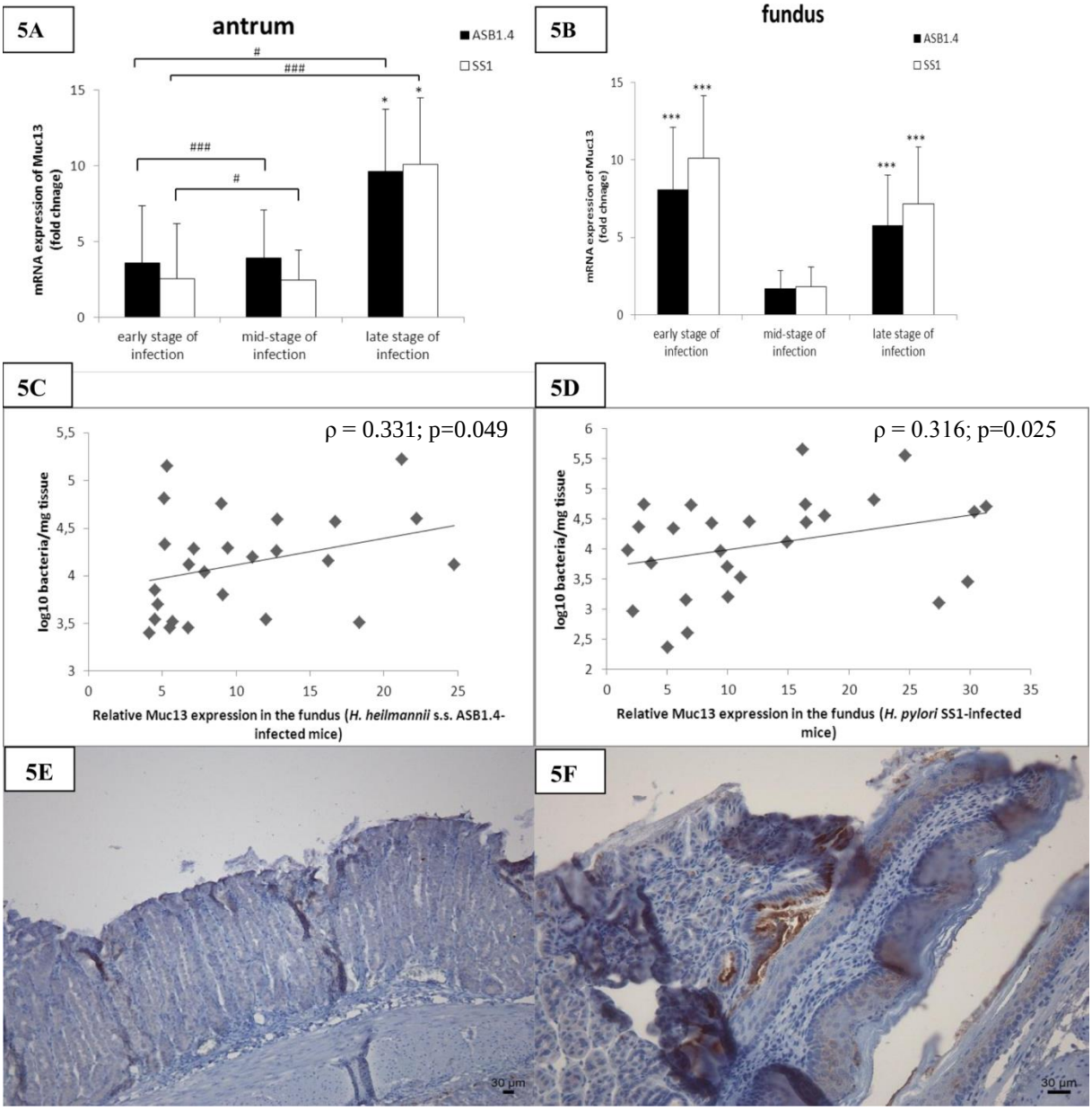

Figure 5: Muc13 expression in the stomach of *Helicobacter*-infected and control mice.

Expression of Muc13 in the antrum (A) and fundus (B) of the stomach of ASB1.4- and SS1-infected BALB/c mice is shown. Data are presented as fold change in gene expression normalized to 3 reference genes and relative to the negative control group which is considered as 1. Data obtained from the time points 1 day, 4 days, 1, 2, 3, 4, and 9 weeks are pooled and designated as "early stage of infection". Data obtained from the time points 12, 16, 20 and 24 weeks are pooled and designated as "mid-stage of infection". Data obtained from the time points 34 and 52 weeks are pooled and designated as "late stage of infection". Data are shown as means + standard deviation. Significant differences in expression level between the infected groups and the negative control group at a certain time frame are indicated by * $p<0.05$ or *** $p<0.001$ (ANOVA). Significant differences in expression level between groups inoculated with *H. heilmannii* ASB1 or *H. pylori* SS1 of different time frames are indicated by # $p<0.05$ or ### $p<0.001$ (ANOVA).(C & D) Correlation analysis between Muc13 mRNA expression and the number of colonizing *Helicobacter* bacteria in the stomach of BALB/c mice. Time-points 4 days, 1 week, 2, 4 and 9 weeks were taken into account. Correlation was measured by Spearman's Rho (ρ,

p<0.05). (E &F) Immunohistochemical analysis of Muc13 expression (brown) in the fundus of the stomach of a control mouse (E; Bar = 30 µm) and a mouse infected with *H. heilmannii* ASB1.4 for 4 weeks (F; Bar = 30 µm).

H. heilmannii induces reduced expression of markers for gastric acid secretion by parietal cells in the fundus of the stomach.

To determine whether *Helicobacter* colonization has an impact on gastric acid secretion, expression of different markers was analyzed (Jain et al., 2006). Quantitative RT-PCR analysis showed a clear down-regulation in the expression of H^+/K^+-ATPase α and β proton pump subunits in the fundus of *H. heilmannii*- and *H. pylori*-infected mice at 52 weeks post-infection (Figures 6A & 6B). Compared to the control animals with mRNA expression level set to 1, the mean relative expression of H^+/K^+-ATPase α was 0.24 ± 0.14 and 0.2 ± 0.07 for ASB1.4- and SS1-infected animals, respectively. The H^+/K^+-ATPase β relative mean expression was 0.23 ± 0.15 and 0.22 ± 0.14 for ASB1.4- and SS1-infected animals, respectively. The KCNQ1 potassium channel, which co-localizes with the proton pump at the apical membrane, has been proposed to be responsible for K^+ conductance associated with acid secretion (Grahammer et al., 2001). Its expression level was normal until 34 weeks post-infection, but was significantly downregulated (ASB1.4: 0.2 ± 0.11; SS1: 0.23 ± 0.07) at 52 weeks post-infection in the fundus of *Helicobacter*-infected mice compared to the control group (Figure 6C). Reduction in the fundic expression of H^+/K^+-ATPase α and β proton pump subunits and the KCNQ1 potassium channel suggests reduced gastric acid secretion by parietal cells. A loss of parietal cells could clearly be visualized by immunohistochemical staining in the fundus close to the forestomach/stomach transition zone of the stomach of ASB1.4- and SS1-infected mice (Figure 6D, 6E & 6F). Quantification of parietal cells in 5 randomly chosen High Power Fields in the fundus of the stomach of animals infected for 52 weeks also showed a clear reduction of parietal cells in ASB1.4- and SS1-infected mice compared to the controls (Figure 6G).

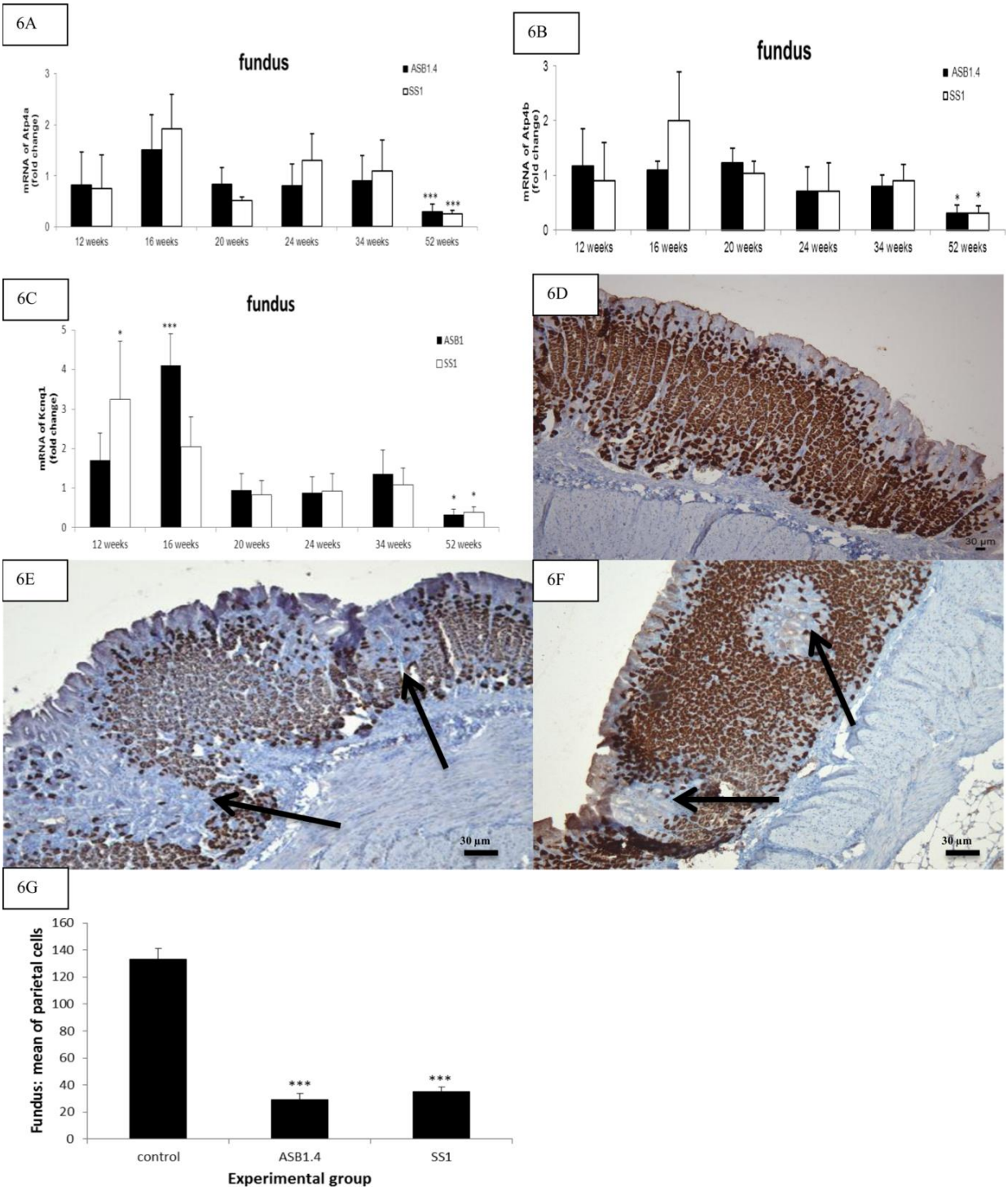

Figure 6: Analysis of parietal cells in the fundus of the stomach of *Helicobacter*-infected and control mice. Expression levels of Atp4a (A), Atp4b (B) and Kcnq1 (C) in the fundus of the stomach of *H. heilmannii* ASB1.4- and *H. pylori* SS1-infected mice are shown. Data are presented as fold change in gene expression normalized to 3 reference genes and relative to the negative control group which is considered as 1. Significant differences in expression level between the infected groups and the negative control group at a certain time point are indicated by * $p<0.05$ or *** $p<0.001$ (ANOVA).

(D, E & F) Immunohistochemical staining for the hydrogen potassium ATPase. (D) ATPase staining of the fundus of a sham-inoculated mouse. (E & F) Loss of parietal cells (arrows) was seen in the fundus of the stomach of a mouse infected with *H. heilmannii* ASB1.4 for 52 week, (Bar = 30 µm).
(G) The mean numbers of parietal cells in the fundus of the stomach from mice infected with *Helicobacter* for 52 weeks and control mice are shown. The number of parietal cells in each stomach was determined by counting ATPase-positive cells in 5 randomly chosen High Power Fields at the level of the gastric pits. Significant differences between *Helicobacter*-infected and control animals are indicated by *** (ANOVA, $p < 0.001$).

Markers for metaplastic progression are upregulated in the fundus of the stomach in response to chronic infection with *H. heilmannii.*

Loss of parietal cells can lead to mucous metaplasia. To investigate whether metaplastic lineages were found in the stomach, mRNA expression of different markers for metaplastic progression was analyzed (Weis et al., 2013). From 16 weeks post-infection onwards, Muc4 expression was upregulated in the fundus of the stomach of *Helicobacter*-infected animals compared to the controls and its expression remained high until the end of the study at 52 weeks post-infection (Figure 7A). The mRNA expression level of Dmbt1, which has a function in epithelial cell differentiation and gastric mucosal protection (Conde et al., 2007), was highly increased between 20 and 52 weeks post-infection in the fundus of *H. heilmannii*- and *H. pylori*-infected mice (Figure 7B). Similar results were found for the polymeric immunoglobulin receptor (PIgR), which is involved in the transport of immunoglobulin A across mucosal membranes, from the basolateral aspect of epithelial cells to the luminal surface. PIgR is highly expressed in intestinal epithelial cells but is not present in the normal gastric mucosa (Gologan et al., 2008). During this *in vivo* experiment, its expression level was highly upregulated from 20 weeks post-infection onwards in the fundus of mice infected with both species (Figure 7C). Expression of Tff2 was significantly increased at 52 weeks post-infection in the fundus (ASB1.4: 6.49 ± 3.32, SS1: 3.44 ± 1.49) (Supplementary figure 1F). Muc4, Dmbt1, PIgR and Tff2 have been described to be related to SPEM with chronic inflammation (Weis et al., 2013; Nomura et al., 2004). A PAS Alcian blue staining showed at 34 and 52 weeks post-infection evidence for mucous metaplasia in which metaplastic columnar glands, mainly initiating from the transition zone junction between the forestomach and glandular epithelium along the lesser curvature (Figure 7G) and to a lesser extent in the antrum (Figure 7E) and fundus (Figure 7D) of the stomach of *Helicobacter*-infected mice. Quantification of blue-stained metaplastic cells in 5 randomly chosen High Power Fields in the forestomach/stomach transition zone also highlighted the presence of mucous metaplasia

in the stomach of animals infected for 52 weeks with ASB1.4 and SS1 but not in the non-infected controls (Figure 7H).

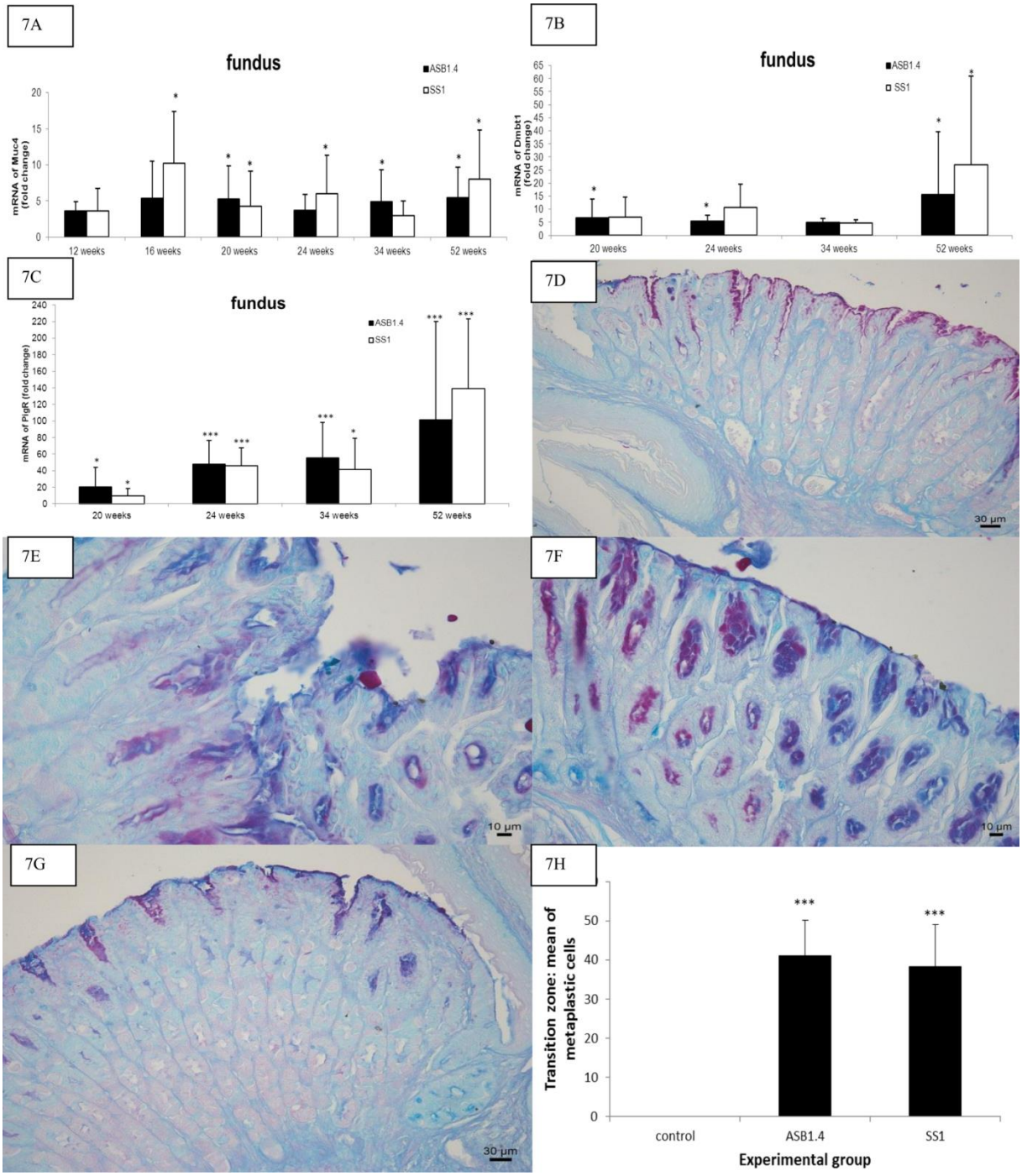

Figure 7: Determination of mucous metaplasia in the fundus of the stomach of *Helicobacter*-infected.

mRNA expression levels of Muc4 (A), Dmbt1 (B) and PigR (C) in the fundus of the stomach of *H. heilmannii* ASB1.4- and *H. pylori* SS1-infected mice are shown. Data are presented as fold change in gene expression normalized to 3 reference genes and relative to the negative control group which is considered as 1. Data are shown as means + standard deviation. Significant differences in expression level between the infected groups and the negative control group at a certain time point are indicated by * $p<0.05$ or *** $p<0.001$ (ANOVA).

(D) PAS-Alcian Blue staining of the the forestomach/stomach transition zone of a sham-inoculated mouse. PAS-Alcian Blue staining of the antrum (E) and the fundus (F) of the stomach of a mouse infected with *H. heilmannii* ASB1 for 24 weeks. (G) PAS-Alcian Blue staining of the forestomach/stomach transition zone of a mouse infected with *H. heilmannii* ASB1 for 52 weeks. (7E & 7F: Bar = 10 µm; 7D & 7G: Bar = 30 µm). Metaplastic columnar cells, mainly initiating from the transition zone junction between the forestomach and glandular epithelium along the lesser curvature (G) and to a lesser extent in the antrum (E) and fundus (F) of the stomach of *Helicobacter*-infected mice, are indicated in blue.

(H) The mean numbers of metaplastic cells in the forestomach/stomach transition zone of the mice infected with *Helicobacter* for 52 weeks and control mice are shown. The number of metaplastic cells was determined by counting blue cells in 5 randomly chosen High Power Fields after staining with PAS Alcian blue. Significant differences between *Helicobacter*-infected and control animals are indicated by *** (ANOVA, $p < 0.001$).

Discussion

In BALB/c mice infected with *H. heilmannii* ASB1.4 and *H. pylori* SS1 for 52 weeks, MALT-lymphoma-like lesions were observed in a narrow zone in the fundus near the forestomach/stomach transition zone. These pathological lesions might eventually lead to gastric MALT lymphoma (O'Rourke et al., 2004a). The risk to develop MALT lymphoma has been suggested to be higher in humans suffering from a NHPH gastritis than those infected with *H. pylori* (Flahou et al., 2010). Gastric MALT lymphoma is characterized by a strong proliferation of B-lymphocytes which may be dependent on Th2-type cytokines (Flahou et al., 2010, Flahou et al., 2012). Experimental NHPH infections have indeed been shown to evoke a Th2-polarized response (Joosten et al., 2013, Flahou et al., 2012), suggesting that Th2-prone BALB/c mice (Enno et al., 1998) infected with NHPH can be seen as a critical model for the development of MALT lymphoma induced by NHPH.

It has been demonstrated that *H. pylori* strains mainly stimulate Th1 responses both in humans and in mouse models (Robinson et al., 2005). However, as an exception, the *H. pylori* strain SS1 does not cause a significant upregulation of IFN-γ expression, a signature Th1 marker, in either in BALB/c or in C57BL/6 mice. Nevertheless, in common with other NHPH, it elicits a Th2 response in mice (Crabtree et al., 2002; Thompson et al., 2004). This may explain the development of MALT lymphoma-like lesions in the stomach seen in this and other studies (Thompson et al., 2004). Typical for *H. pylori* strains inducing MALT lymphoma is that they lack genes encoding major virulence factors such as a functional CagPAI, Bab and Sab adhesins (Thiberge et al., 2010). Compared to its parental strain (PMSS1), *H. pylori* SS1 indeed lacks a functional CagPAI (Crabtree et al., 2002). This strain also doesn't bind to the glycan structures Le^b and sLe^x expressed by human gastric mucins (unpublished data).

Binding to Le^b and sLe^x has been shown to be mediated by the *H. pylori* BabA and SabA adhesins, respectively (Linden et al., 2002; Linden et al., 2009), suggesting that SS1 doesn't express these adhesins. These virulence factors as well as a functional Cag PAI are also absent in *H. heilmannii* and other NHPH (Smet et al., 2013; Vermoote et al., 2011; Schott et al., 2011; Arnold et al., 2011).

In this study, *H. heilmannii* ASB1.4 and *H. pylori* SS1 colonized both the antrum and fundus of the stomach but with a higher colonization density seen in the antrum. This is similar to what has been described in human patients. Indeed, in humans infected with NHPH, colonization mainly occurs in the antrum of the stomach but these bacteria may be found in the fundus as well, which has also been described for *H. pylori* (Haesebrouck et al., 2009). In the present study, *H. heilmannii*-infected BALB/c mice showed higher colonization rates in the antrum and fundus of the stomach compared to *H. pylori*-infected mice. This indicates that the capacity of ASB1.4 to persist in the stomach of BALB/c mice is higher than that of SS1 which showed a reduction in colonization during the later stages of infection. This latter finding has also been reported by Schmitz et al. (2009). DNA from *H. heilmannii* ASB1.4 and *H. pylori* SS1 was also found in the duodenum. Since both species have been linked to duodenal ulcer disease (Haesebrouck et al., 2010), it remains to be elucidated whether they are able to colonize the duodenum or whether the qRT-PCR just picked-up DNA from bacteria colonizing the stomach.

The expression of MUC1, MUC5AC and MUC6 in the human gastric epithelium in relation to *H pylori* colonization has been previously investigated showing that *H. pylori* interacts with epithelial cells that produce MUC1 and MUC5AC by binding to Le^b and sLe^x expressed by these mucins. This indicated that MUC1 and MUC5AC, but not MUC6, plays a role in the colonization of *H pylori* to the gastric mucosa (Linden et al., 2002; Linden et al., 2009). On the contrary, in this study, a clear upregulation of Muc6 but not Muc5AC and Muc1 was seen in the stomach of *H. heilmannii* ASB1.4-infected BALB/c mice in the first 9 weeks post-infection. The pathway regulating gastric MUC6 expression in response to *H. heilmannii* infection in the human stomach as well as the bacterial factors involved are unknown and need further investigation. During this early stage of infection, the increased expression of Muc6 in the antrum was also positively correlated with the number of *H. heilmannii* bacteria. Since Muc6 is expressed by the glands and, unlike *H. pylori* (Linden et al., 2002), NHPH are mainly localized in the deep glands of the gastric mucosa (Haesebrouck et al., 2009; Flahou et al., 2010; Flahou et al., 2012), the potential role of Muc6 in *H. heilmannii* colonization is

suggested and needs to be further unraveled. Whether Muc6 plays a role in colonization of *H. pylori* strains lacking the BabA and SabA adhesins, such as the SS1 strain, needs also further investigation.

Another interesting finding seen during *H. heilmannii* ASB1.4 as well as *H. pylori* SS1 infection was the increased mRNA expression of Muc13 in the stomach. MUC13 has been shown to be highly expressed in human gastric cancer (Sheng et al., 2011) but an increased mRNA expression in the early stages of *Helicobacter* colonization has so far never been described. The positive correlation found between the increased Muc13 expression and the increased number of *Helicobacter* bacteria in the fundus of the stomach during the first 9 weeks of infection, suggests a potential role for Muc13 in *Helicobacter* colonization. The expression level of Muc13 remained upregulated until 52 weeks post-infection. It has been described that a sustained elevation in expression of cell-surface mucins may promote the transition from chronic inflammation to cancer (Sheng et al., 2011). How Muc13 influences the *Helicobacter* colonization process is unknown and needs further investigation.

In this study, mRNA expression of several markers for gastric acid secretion by parietal cells was significantly reduced at 52-weeks post-infection in the fundic epithelium of *H. heilmannii*-infected mice suggesting loss of parietal cell function. A clear loss of parietal cells was indeed shown by immunohistochemical staining. Parietal cell loss might eventually lead to the development of mucous metaplasia (Weis et al., 2013). Markers for metaplastic progression into SPEM were indeed found to be upregulated in the fundic region of the stomach during later stages of infection with *H. heilmannii* ASB1.4 and *H. pylori* SS1. Mucous metaplasia in a narrow zone of the fundus near the limiting ridge of the stomach of *H. heilmannii*-infected mice was histologically confirmed. MALT-lymphoma-like lesions were also seen in this region. In gastric cancer, intestinal metaplasia present in the mucosa surrounding low-grade MALT-lymphomas has been described (Lamarque et al., 2006). However, in our study, evidence for intestinal metaplasia, such as *de novo* expression of Muc2 as described in *H. pylori* infection (Mejias-Luque et al., 2010), was not seen. It remains, therefore, to be determined whether SPEM may further differentiate into intestinal metaplasia and in worst case scenario into dysplasia in mice kept for longer than 1 year after experimental infection with *H. heilmannii*.

Taken together the results of histopathology and quantitative RT-PCR, the present experimental infection study in BALB/c mice illustrates that infection with *H. heilmannii*

induced severe gastric pathology that progressed into MALT lymphoma like lesions and SPEM as well as changes in the expression of Muc6 and Muc13 in the stomach.

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TABLE S1: List of primers used in quantitative RT-PCR for gene expression.

Primer*	Sequence	Reference
Hh_FWQ	5'-CTT TCT CCT GGT GAA GTG ATT CTC-3'	1
Hh_RVQ	5'-GCT GTA CCA GAG GCA ATG TCC AAG-3'	1
Hp_F1	5'- AAA GAG CGT GGT TTT CAT GGC G -3'	2
Hp_R1	5'- GGG TTT TAC CGC CAC CGA ATT TAA -3'	2
H2afz-F	5'-GGT ATC ACC CCT CGT CAC TT-3'	2
H2afz-R	5'-TCA GCG ATT TGT GGA TGT GT-3'	2
HPRT-F	5'-CAG GCC AGA CTT TGT TGG AT-3'	2
HPRT-R	5'-TTG CGC TCA TCT TAG GCT TT-3'	2
PPIA-F	5'-AGC ATA CAG GTC CTG GCA TC-3'	2
PPIA-R	5'-TTC ACC TTC CCA AAG ACC AC-3'	2
Muc1-F	5'-GGT TGC TTT GGC TAT CGT CTA TTT-3'	This study
Muc1-R	5'-AAA GAT GTC CAG CTG CCC ATA-3'	This study
Muc2-F	5'-GTC TGC CAC CTC ATC ATG GA-3'	This study
Muc2-R	5'-CAG GCA AGC TTC ATA GTA GTG CTT-3'	This study
Muc4-F	5'-TCT TTC TGT CTC AAC TGT TGA ATC AGA-3'	This study
Muc4-R	5'-CGT GGC CAG GAT GTC AAA C-3'	This study
Muc6-F	5'-TGC TCC CAG AAT GAG TAC TTC GA-3'	This study
Muc6-R	5'-CAG AGG TGG AAC TGT GAA ACT CAG T-3'	This study
Muc13-F	5'-GCC AGT CCT CCC ACC ACG GTA-3'	This study
Muc13-R	5'-CTG GGA CCT GTG CTT CCA CCG-3'	This study
Muc5b-F	5'-CAG ATC CAT CCA TCC CAT TTC T-3'	This study
Muc5b-R	5'-TAT CTG ACT ACC ACT TGT TGA TGT GAC T-3'	This study
Tff1-F	5'-ATG CTG GCC TTC GGC AGC CTT GCC-3'	This study
Tff1-R	5'-GCA ACC TCT CTC CGT GCA CTG CTG-3'	This study
Tff2-F	5'-TTG GGA CTG CAT GCT CTG GTA GAG-3'	This study
Tff2-R	5'-AGG GAC CCC AGC GAC GCT AGA GTC-3'	This study
H+, K+-ATPase α-F	5-TGC TGC TAT CTG CCT CAT TG-3'	3
H+, K+-ATPase α-R	5'-GTG CTC TTG AAC TCC TGG TAG-3'	3
H+, K+-ATPase β-F	5'-AAC AGA ATT GTC AAG TTC CTC-3'	3
H+, K+-ATPase β-R	5'-AGA CTG AAG GTG CCA TTG-3'	3
Dmbt1 F	5'-ACC TCC TCA CGG TGC TAC AG-3'	3
Dmbt1 R	5'-GCT TCT TCA CAT CCT CCA CTG-3'	3
PigR F	5'-GAT TTG GGA GGC AAT GAC AAC-3'	3
PigR R	5'-GCT TTC TTG GAT TCT TCT GGC-3'	3

Kcnq1 F	5'-AGA TGC GGT GAA CGA GTC-3'	3
Kcnq1 R	5'-CAG GAG GCG ATG GTC TTC-3'	3
Ckb F	5'-GAT CTG AGC AGC CAC AAC-3'	3
Ckb R	5'-GTC TAC GCC AGT CTG AAT G-3'	3
Nmyc F	5'-ACC TCC GGA GAG GAT ACC TTG AG-3'	3
Nmyc R	5'-CAT AGT TGT GCT GCT GAT GGA TGG-3'	3

*Primers used for measuring mRNA expression of Muc5AC were purchased from QIAGEN Cat.no.:Qt01196006

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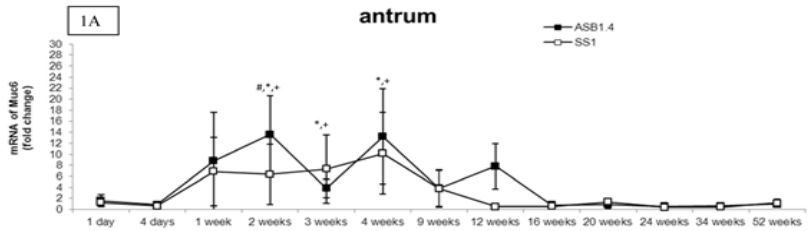
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antrum
ASB1.4
SS1
mRNA of Muc6 (fold change)
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4 days
1 week
2 weeks
3 weeks
4 weeks
9 weeks
12 weeks
16 weeks
20 weeks
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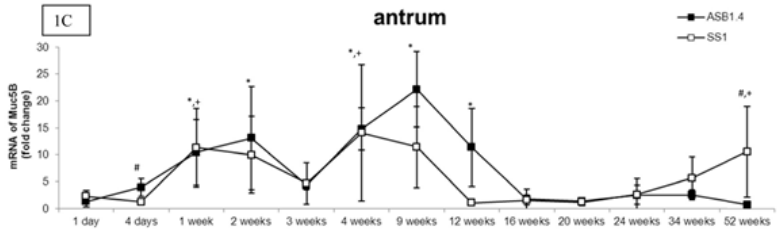
34 weeks
52 weeks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mRNA of Muc5B (fold change)
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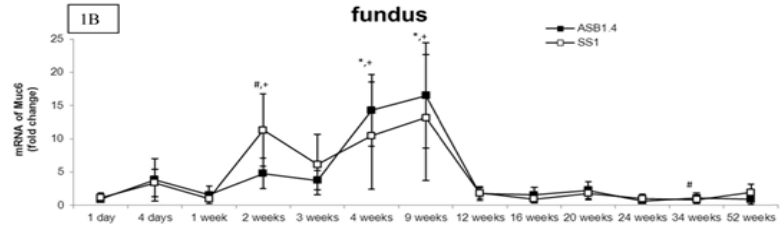
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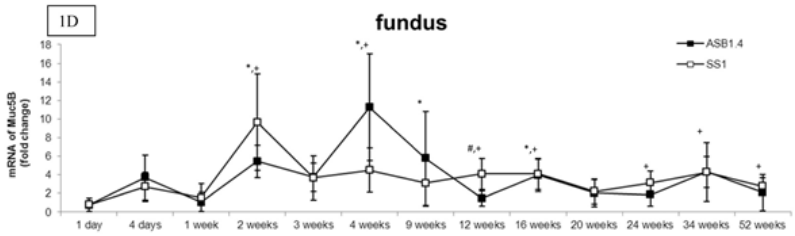
34 weeks
52 weeks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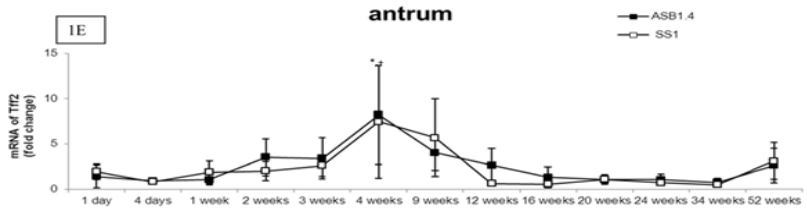
52 weeks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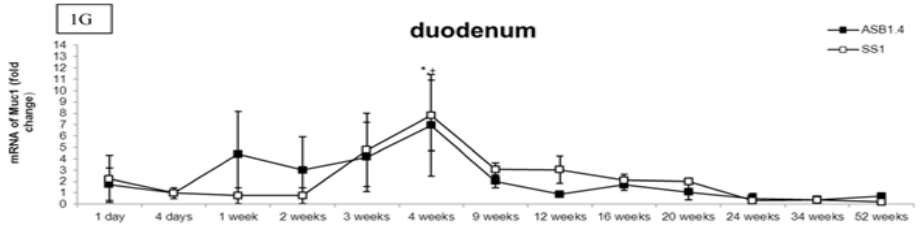
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SS1
mRNA of Muc1 (fold change)
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2 weeks
3 weeks
4 weeks
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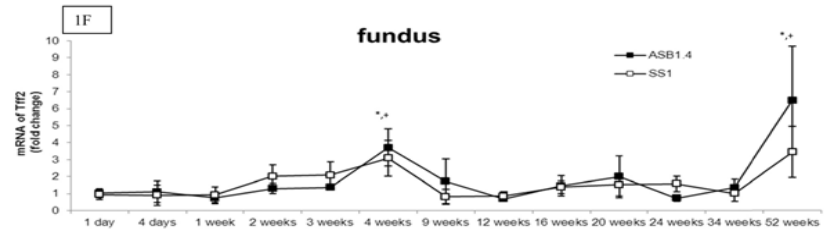
12 weeks
16 weeks
20 weeks
24 weeks
34 weeks
52 weeks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fundus
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SS1
mRNA of Tff2 (fold change)
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4 days
1 week
2 weeks
3 weeks
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12 weeks
16 weeks
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52 weeks

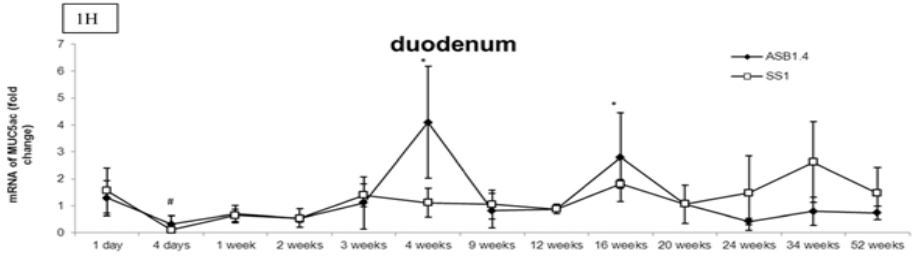
1H
duodenum
ASB1.4
SS1
mRNA of MUC5ac (fold change)
1 day
4 days
1 week
2 weeks
3 weeks
4 weeks
9 weeks
12 weeks
16 weeks
20 weeks
24 weeks
34 weeks
52 weeks

Figure. S1: mRNA expression levels of Muc6, Muc5B and Tff2 in the stomach and Muc1 and Muc5AC in the duodenum of *H. heilmannii* ASB1.4- and *H. pylori* SS1-infected mice. Expression levels of Muc6 in the antrum (A) and fundus (B), Muc5B in the antrum (C) and fundus (D), Tff2 in the antrum (E) and fundus (F) and Muc1 (G) and Muc5AC (H) in the duodenum are shown. Data are presented as fold change in gene expression normalized to 3 reference genes and relative to the negative control group which is considered as 1. Data are shown as means + standard deviation. Significant differences in expression level between groups inoculated with *H. heilmannii* ASB1 and *H. pylori* SS1 at a certain time-point are indicated by # (Mann-Withney U test, $p<0.05$). Significant differences in expression level between the infected groups and the negative control group at a certain time point are indicated by * for the ASB1.4-infected mice and + for SS1-infected mice (Mann-Withney U test, $p<0.05$).

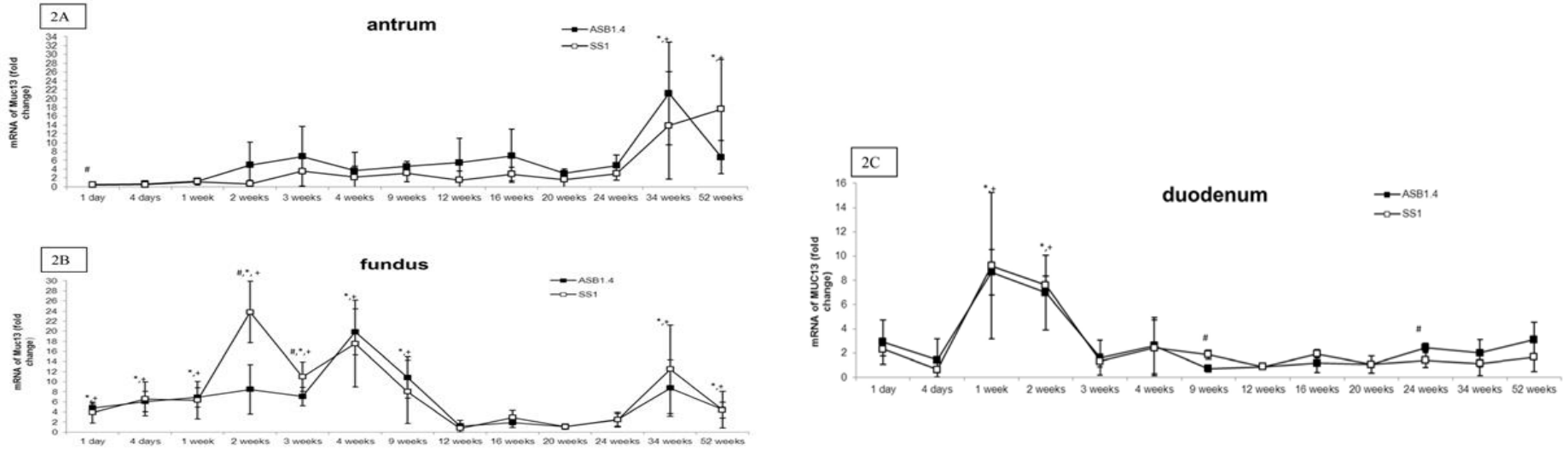

Figure S2: mRNA expression level of Muc13 in the stomach and duodenum of *H. heilmannii* ASB1.4- and *H. pylori* SS1-infected mice. Expression levels of Muc13 in the antrum (A) and fundus (B) of the stomach and the duodenum (C) are shown. Data are presented as fold change in gene expression normalized to 3 reference genes and relative to the negative control group which is considered as 1. Data are shown as means + standard deviation. Significant differences in expression level between groups inoculated with *H. heilmannii* s.s. ASB1.4 and *H. pylori* SS1 at a certain time-point are indicated by # (Mann-Withney U test, $p<0.05$). Significant differences in expression level between the infected groups and the negative control group at a certain time point are indicated by * for the ASB1.4-infected mice and + for SS1-infected mice (Mann-Withney U test, $p<0.05$).

The *Helicobacter heilmannii hofE* and *hofF* genes are essential for colonization of the gastric mucosa and are involved in IL-1β-induced gastric MUC13 expression

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Abstract

Helicobacter heilmannii is a zoonotic bacterium associated with gastric disease in humans. We recently showed that *H. heilmannii* binds to human gastric mucins and epithelial cells and highlighted a potential role for the murine Muc13 mucin in gastric *Helicobacter* colonization. The aims of this study were to investigate the role of the *H. heilmannii hof* gene locus encoding HofH/F/E/G/C/D in adhesion to the gastric mucosa and induction of increased gastric Muc13 expression.

Bacterial *hof* gene and host gene expression experiments, *Helicobacter* binding assays and experimental infection studies in mice were performed. *H. pylori* and its Δ*hofF* mutant were included for comparison.

H. heilmannii strains lacking HofE or HofF showed a clear decrease in binding to gastric mucins and epithelial cells as well as a lower gastric colonization level in the stomach of Balb/c mice at 4 and 9 weeks post-infection compared to the *H. heilmannii* wildtype strain. Interestingly, *H. heilmannii* Δ*hofE* and Δ*hofF* and *H. pylori* Δ*hofF* did not induce an increased expression of MUC13 in human gastric epithelial cells and of Muc13 in the stomach of mice. Finally, we demonstrated that IL-1β is induced in the stomach as a response to *Helicobacter* colonization which on its turn is involved in the expression of MUC13/Muc13 in the gastric epithelium.

These novel results in *Helicobacter* research identified *H. heilmannii* HofE and HofF as adhesins and suggest an important role of *H. heilmannii* HofE and HofF and *H. pylori* HofF in IL-1β-induced gastric MUC13/Muc13 expression.

Introduction

Helicobacter pylori colonizes the stomach of more than half of the human population. Infection with this agent causes a wide variety of gastric disorders, including gastritis, ulcers and cancer (Montecucco et al., 2001). However, gastric disease in humans has also been associated with other, long spiral-shaped NHPH naturally colonizing the stomach of domesticated animals, such as cats, dogs and pigs (Haesebrouck et al., 2009). One of the most predominant *Helicobacter* species in the feline stomach is *H. heilmannii* (Haesebrouck et al., 2009; Smet et al., 2012). This *Helicobacter* species is highly prevalent in healthy cats as well as animals showing chronic vomiting (Haesebrouck et al., 2009). Most likely, transmission of *H. heilmannii* to humans occurs through direct or indirect contact between cats and humans (Haesebrouck et al., 2009). In humans, this pathogen has been associated with gastritis, peptic and duodenal ulcers and MALT lymphoma. The risk of developing MALT lymphoma is higher after infection with a NHPH species, such as *H. heilmannii*, than with *H. pylori* (Haesebrouck et al., 2009; Liu et al., 2014; Joosten et al., 2013; O'Rourke et al., 2004; Van Den Bulck et al., 2005; Baele et al., 2009).

Information on how *H. heilmannii* colonizes the human gastric mucosa still remains scarce. The human gastric mucosa is covered by a layer of continuously secreted mucus that washes away trapped particles. It is the first barrier *Helicobacter* encounters and consists of heavily glycosylated secreted mucins (MUC) (Linden et al., 2002; McGuckin et al., 2011). In a healthy human stomach, MUC1, MUC5AC and MUC6 are the major mucins covering the gastric mucosa. The membrane-associated MUC1 and secreted MUC5AC are expressed by the surface epithelium, whereas MUC6 is secreted by glandular cells (Linden et al., 2002; McGuckin et al., 2011; Sheng et al., 2012). In a recent one-year experiment, Balb/c mice experimentally infected with *H. heilmannii* developed severe gastric pathologies that progressed into MALT-lymphoma like-lesions and SPEM as well as changes in the expression pattern of gastric mucins (Liu et al., 2014). Of particular interest was the increased expression of the murine Muc13 mucin already in the early stages of *H. heilmannii* colonization. This novel phenomenon was also observed during *H. pylori* infection (Liu et al., 2014). Human MUC13 and murine Muc13 are transmembrane mucins that are most abundant in the intestinal tract. In the healthy stomach, the expression level is very low (Sheng et al., 2011). The positive correlation between the increased Muc13 expression and the increased number of *H. heilmannii* and *H. pylori* bacteria in the stomach of mice during the acute stage of infection highlights a potential role for Muc13 in *Helicobacter* colonization (Liu et al.,

2014).

Adherence to the gastric mucosa is widely assumed to play an important role in the initial colonization of *Helicobacter* in the stomach (McGuckin et al., 2011). The vast majority of *H. pylori* remains in the mucus layer by adherence to mucins, while a small percentage binds to the epithelial cell surfaces underneath the mucus layer (Linden et al., 2002; Appelmelk et al., 2000). More recently, we showed that *H. heilmannii* also binds to human mucin samples as well as to gastric epithelial cells (Smet et al., 2014). In general, *H. pylori* and *H. heilmannii* have a higher binding capacity to mucins at pH2 and to epithelial cells at pH7 (unpublished results; Skoog et al., 2012). *H. pylori* is equipped with a large set of OMPs, which role in the adhesion process has been well-studied. The *H. pylori* OMPs BabA/B (HopS/T), SabA (HopP), AlpA/B (HopB/C), OipA (HopH), HopZ, HopQ and HomB have been identified as adhesins (Oleastro et al., 2013; Ilver et al., 1998; Mahdavi et al., 2002; Alm et al., 2000; Kavermann et al., 2003; Odenbreit et al., 1999). Based on the available genome sequence of *H. heilmannii*, this microorganism also harbours a large set of OMPs which is in agreement with the approximately 64 well annotated OMPs described in *H. pylori* (Alm et al., 2000). Interestingly, it lacks all *H. pylori* OMPs identified as adhesins so far, indicating that other OMPs might be involved in binding (Smet et al., 2013). *H. heilmannii* contains a ca. 10kb locus encoding homologs of 6 *H. pylori* Hof OMP proteins (HofH/F/E/G/C/D, Fig. 1). This *hof* locus is also present in other zoonotic NHPH, such as *H. felis* (Figure 1, Smet et al., 2014).

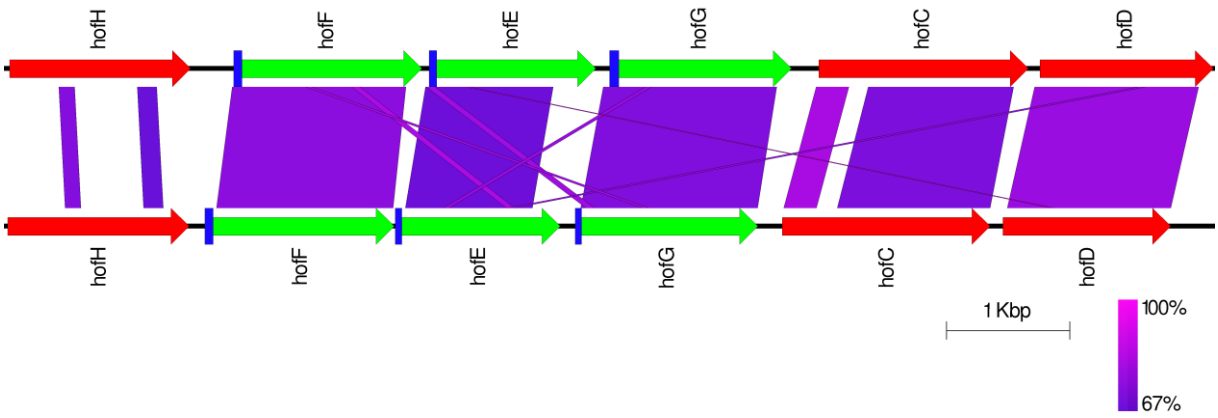


Figure 1: Schematic representation of the *hof* gene locus of the *H. heilmannii* ASB1 strain (above; 9780 bp; EMBL genome accession number CDMK00000000) and *H. felis* CS1 (ATCC49179) strain (below; 9451 bp; NCBI genome accession number NC_014810). Blast comparisons between these two genomic regions showed an overall nucleotide identity of 78%. This locus harbors the *hofH* (1470 bp (ASB1); 1476 bp (CS1)), *hofF* (1530 bp (ASB1); 1539 bp (CS1)), *hofE* (1356 bp (ASB1); 1341 bp (CS1)), *hofG* (1479 bp (ASB1); 1485 bp (CS1)), *hofC* (1704 bp (ASB1); 1689 bp (CS1)) and *hofD* (1410 bp (ASB1); 1365 bp (CS1)) genes. A N-terminal signal sequence was predicted by the bioinformatics tools SignalP 3.0 and Phobius for HofF, HofE and

HofG. The DNA fragments encoding the N-terminal signal sequences for these Hof OMPs are highlighted as blue rectangles.

On the contrary, the *hof* genes of *H. pylori* are scattered across its genome (Smet et al., 2013; Schott et al., 2011). A signature tagged mutagenesis approach by Kavermann and his colleagues (2003) showed that the *H. pylori* HofF protein is essential for gastric colonization. They even suggested that this OMP might have a function as adhesin (Kavermann et al., 2003).

The main aim of the present study was to obtain better insights in the role of the *H. heilmannii hof* gene locus in gastric colonisation and its relationship to the increased gastric Muc13 expression seen in mice during *Helicobacter* colonization. In order to colonize the stomach, *H. pylori* is able to regulate the expression of its *omp* genes in response to environmental host factors (Skoog et al., 2012; de Vries et al., 2001; Huang et al., 2011; Wen et al., 2003). We therefore defined the *H. heilmannii hof* gene expression patterns in response to different conditions reflecting the gastric environment. Subsequently, the role of these Hof proteins in adherence to the gastric mucosa and in gastric human MUC13 and murine Muc13 expression was studied. An *H. pylori* wildtype and Δ*hofF* mutant strain were included for the purpose of comparison. Expression and mutational analyses of the *hof* genes, the *Helicobacter* binding assays and the experimental infection study in mice identified *H. heilmannii* HofE and HofF as adhesins and confirmed the role of *H. pylori* HofF in gastric colonization. These analyses also revealed the importance of *H. heilmannii* HofE and HofF and *H. pylori* HofF in IL-1β-induced gastric MUC13/Muc13 expression.

Materials and Methods

Helicobacter strains and growth conditions.

The highly virulent *H. heilmannii* ASB1 wildtype (wt) strain, isolated from the gastric mucosa of a kitten with severe gastritis, was cultivated biphasically on *Brucella* agar plates at pH5 under microaerobic conditions at 37°C (Smet et al., 2012). The *H. pylori* SS1 strain (Liu et al., 2014), the *H. pylori* STM138*ΔhofF* strain and its corresponding P1 wildtype strain (Kavermann et al., 2003) were all grown microaerobically on blood agar plates (Oxoid) at 37°C (Liu et al., 2014; Kavermann et al., 2003). The SS1 strain is a mouse-adapted strain and used in this study for *in vivo* purposes only. The STM138*ΔhofF* is able to colonize rodents as shown before and was used in *in vitro* and *in vivo* experiments, while its parental P1 strain was only enclosed in the *in vitro* cell experiments (Kavermann et al., 2003).

***In vitro* exposure of *H.heilmannii* to different conditions reflecting the host environment.**
Liquid cultures of *H. heilmannii* ASB1wt (optical density at 600 nm (OD_{600}): 0.5-1.0) were exposed to different pH environments, high temperature or H_2O_2. To induce acid stress, the pH in the *Helicobacter* growth medium was adjusted to 2 (pH in the gastric lumen) using concentrated hydrochloric acid. To simulate pH conditions in the gastric glands and surface epithelium under the mucus layer, *H. heilmannii* was exposed to pH7 using buffered conditions. Oxidative stress was simulated by adding 1% H_2O_2 to the growth medium. All these cultures were incubated microaerobically at 37°C. To cause temperature stress, another gastric environmental stress condition, bacterial cultures were incubated microaerobically at 42°C instead of 37°C. All experiments were performed in triplicate. Bacterial samples were harvested after 30 min and 1h of incubation at pH2, 42°C or in the presence of H_2O_2. Samples from bacterial cultures that were exposed to pH7 were collected after 24h. Samples at normal growth conditions (pH5) and prior to exposure to the different environmental conditions were included as controls. From all collected samples, *Helicobacter* RNA was extracted as described before (Liu et al., 2014).

Co-cultivation of *Helicobacter* with gastric mucins and epithelial cells.
Mucin samples containing MUC6 and MUC5AC were collected from one tumor sample (labelled as human 1) and two samples with normal histology (labelled human 2 and 3). The samples with normal histology were obtained after informed consent and approval of the local ethical committee (Lund University Hospital, Lund, Sweden), whereas the mucins from the tumor sample was from our well characterized mucin library, and the sample was collected in 1983 at the IMIM-Hospital del Mar, Barcelona, Spain (before the hospital had an ethics comitté). Rinsed and frozen human stomach tissue pieces were drenched in 10 mM sodium phosphate buffer (pH 6.5) containing 0.1 mM phenylmethylsulphonylfluorid (PMSF). The tissue was placed into five volumes of extraction buffer (6M guanidinium chloride, 5mM ethylenediaminetetraacetic acid (EDTA), 10mM sodium phosphate buffer (pH 6.5)) containing 0.1 mM PMSF, dispersed using a Dounce homogenizer (four strokes with a loose pestle) and stirred slowly at 4°C overnight. The insoluble material was removed by centrifugation at 23,000 × g for 50 min at 4°C (Beckman JA-30 rotor). Supernatants were dialyzed against ten volumes of extraction buffer for 24h and filled up to 26 ml with extraction buffer. Cesium chloride was added to the samples to give 1.39 g/ml starting density

by gentle stirring. The samples were centrifuged at 40,000 × g for 90h at 15°C. The fractions were collected from the bottom of the centrifuge tubes with a fraction collector. The dialyzed purified mucin samples were diluted in PBS to get 100 µg/ml concentration. *H. heilmannii* ASB1wt was harvested from biphasic *Brucella* agar plates and cultured in *Brucella* broth containing 20% fetal bovine serum (FBS) at a starting concentration of OD_{600}= 0.25 in the presence of dialyzed mucin. As a control for normal proliferation, bacteria were cultured with phosphate buffered saline (PBS) without mucin. Bacteria were grown in 96-well plates for 24h at 37^{o}C under microaerophilic conditions. Thereafter, the bacterial culture was treated with RNAprotect Bacteria Reagent (Qiagen) according to the manufacturer's instructions and further used for RNA extraction (Liu et al., 2014).

For co-cultivation of *Helicobacter* with epithelial cells, human gastric epithelial MKN7 (Riken Cell Bank, Japan) cells, grown as described before (Linden et al., 2002) were seeded in antibiotic free medium (pH7) at a concentration of 10^4 cells/ml on cover slips in 24-well plates and incubated overnight at 37°C. Then, cells were washed and 500 µl of a bacterial suspension (*H. heilmannii* ASB1wt at a concentration of $5x10^8$ viable bacteria/ml cell culture medium (pH7)) was added to the cells and further incubated for 24h at 37°C under microaerobic conditions. *Helicobacter* strains incubated in the absence of cells were included as control. After 3 washing steps, the epithelial cells with bound bacteria were detached from the surface of the microtitre plates and further used for RNA extraction (Liu et al., 2014).

Quantitative RT- PCR for *hof* gene expression.

The bacterial RNA concentration was determined by measuring the optical density at 260 nm with a spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Each sample was DNase treated. The cDNA was synthesized from 1 µg RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad). Quantitative RT-PCR was carried out for measuring gene expression levels of *H. heilmannii hofC, hofD, hofE, hofF, hofG* and *hofH*. The *H. heilmannii atpA* and *efp* genes were included as housekeeping genes. Primer sequences are shown in Table S3. The quantitative PCR reactions were performed as described before (Liu et al., 2014). No-template-control reaction mixtures were included and all samples were run in duplicate. The Ct-values were normalized to the geometric mean of the Ct-values from the reference genes (*H. heilmannii atpA* and *efp*). Results were expressed as fold changes of *hof* mRNA expression in bacteria exposed to different environmental conditions, mucins or epithelial cells relative to *hof* mRNA expression levels in control bacterial cultures. Fold changes were

calculated using ΔΔCT method (Livak et al., 2001) with mean of Ct-values from three normal bacterial cultures as control.

Creation of mutants by chromosomal insertion mutagenesis.

Analysis of the *H. heilmannii* Hof proteins using the SignalP bio-informatic tool showed that only HofE, HofF and HofG contain a signal sequence. Based on this finding and the results of the *hof* gene expression studies, *H. heilmannii ΔhofE*, *ΔhofF* and *ΔhofG* mutants were constructed as described previously (Kondadi et al., 2013). Briefly, deletion was introduced by allelic exchange using vector pUC119 in which ~300 bp of the 5' –end and 3' –end of the target gene and the chloramphenicol resistance gene from pUOA14 was cloned (Viera and Messing, 1991; Sambrook and Russel, 2001). The constructed deletion vector was then transferred into *E. coli* TOP10 cells. *H. heilmannii* ASB1wt was used for electroporation with 1 µg deletion vector. Mutants were selected (Josenhans et al., 1999) on *Brucella* agar plates (pH 5; 20% FCS; Vitox; 10 mg/ml chloramphenicol) and the site of recombination was verified by PCR and sanger ABI 3730xd sequencing. The expression of the *hof* genes present in the locus was verified in these mutants under normal growth and different environmental conditions (stress, co-incubation with mucins or cells) by RT-PCR as described above.

***In vitro Helicobacter* binding assays to human gastric mucins.**

The *H. heilmannii* ASB1wt and its mutants ASB1*ΔhofE*, ASB1*ΔhofF* and ASB1*ΔhofG* cultured for 24h, were harvested and resuspended in 1% Blocking Reagent for ELISA (Roche, Stockholm, Sweden), containing 0.05% Tween 20 (blocking buffer). Human samples, obtained as described above, were diluted in 4M Guanidinium chloride to 4 mg/ml and coated on 96-well plates (PolySorp; Nunc A/S, Roskilde, Denmark) overnight at 4°C. The wells were then washed with PBS containing 0.05% Tween 20 and blocked for 1h with blocking buffer. After discarding the blocking buffer, the bacteria with an OD600 of 0.1 were diluted 1:10 in blocking buffer containing 10 mM citric acid (pH2 and pH5) and added for 1h to the wells. Thereafter, the 96-well plates were washed and incubated with rabbit anti-*H. pylori* serum (1:1000 dilution in blocking buffer) for 1h at room temperature. Subsequently, the plates were washed and incubated with horseradish peroxidase conjugated anti-rabbit IgG (1:10,000 dilution in blocking buffer) for 1h at room temperature. After the final washing steps, binding was visualized by the addition of 3,3',5,5',-TetraMethylBenzidine substrate (Sigma-Aldrich, Diegem, Belgium), with absorbance read at 450 nm.

***In vitro Helicobacter* binding assays to human gastric epithelial cells.**

The human gastric epithelial cell line MKN7 and the murine gastric surface mucous GSM06 cell line were cultured at 37°C with 5% CO_2 as described before (Linden et al., 2002; Goso et al., 1997).

For the *in vitro* binding assays with *H. heilmannii* ASB1wt and its mutants ASB1*ΔhofE*, ASB1*ΔhofF and* ASB1*ΔhofG*, the cell medium was changed to antibiotic free medium. MKN7 cells and GSM06 cells were seeded at a concentration of 10^4 cells/ml on cover slips in 24-well plates and incubated overnight at 37°C. Then, cells were washed and 500 µl of a bacterial suspension of 10^8 viable bacteria/ml cell medium (pH7) was added to the cells and further incubated for 1h at 37°C under microaerobic conditions. Visualization of bacteria adhering to gastric epithelial cells at pH7 was done by scanning electron microscopy (SEM). For this purpose, coverslips were fixed in 500 µl HEPES fixative (2% paraformaldehyde) and prepared for SEM by routine techniques. The mean number of binding bacteria per cell was calculated by counting spiral-shaped and coccoid *Helicobacter* bacteria attached to 10-20 at random selected cells**.**

***In vitro* MUC13 expression and cytokine analysis.**

Confluent MKN7 cells with low MUC13 expression (Linden et al., 2002) grown in 6 well microtitre plates were inoculated with 10^8 viable ASB1wt, ASB1*ΔhofE*, ASB1*ΔhofF*, ASB1*ΔhofG*, P1 and STM138*ΔhofF* for 24h under microaerobic conditions. Uninfected cells were included as controls. Total RNA was extracted from the cell pellet and cDNA was synthesized. For the measurement of MUC13 mRNA, RT-PCR was performed (Liu et al., 2014; Sheng et al., 2012). Human *GADPH* was included as housekeeping gene. Primers used in these assays are listed in Table S3. Subsequently, the cell culture supernatant was used to quantify cytokine production using a multi-analyte ELISArry^{TM} (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17α, IFNγ, TNFα, GM-CSF; Sabbiosciences). To identify which cytokine is involved in the activation of MUC13 expression, uninfected MKN7 cells were treated with 20 or 40 ng/ml of one of the human recombinant pro-inflammatory cytokines Il-1β, IL-6, IL-8 and tumor necrosis factor (TNF)-α (Mejias-Luque et al., 2008). Total RNA extracted from the cells was then screened for MUC13 expression as described above.

Animals and experimental procedure.

Six-week old SPF, female Balb/c mice, purchased from Harlan NL (Horst, The Netherlands) were housed in individual filter top cages, had free access to water and food (an autoclaved commercial diet (TEKLAD 2018S, containing 18% protein; Harlan) throughout the experiment and were monitored daily. The *in vivo* experimental protocol was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC2014_106, September 2014).

The final concentration of *H. heilmannii* ASB1wt and its mutants ASB1*ΔhofE*, ASB1*ΔhofF*, ASB1*ΔhofG* was adjusted to 7×10^8 viable bacteria/ml. For *H. pylori* SS1 and STM138*ΔhofF,* a concentration of 1×10^9 viable bacteria/ml was used. In total, 90 animals were included and divided in 6 *Helicobacter*-infected groups of 13 animals (ASB1wt, ASB1*ΔhofE*, ASB1*ΔhofF*, ASB1*ΔhofG*, SS1 or STM138*ΔhofF*) and 1 control group of 12 animals. The *Helicobacter*-infected animals were intragastrically inoculated 3 times at 2 days interval with 300 µL of a *Helicobacter* suspension. The control group was inoculated with *Brucella* broth (pH 5). At 4 (acute stage of infection) and 9 (chronic stage of infection) weeks post-infection, animals (5 animals from each group at 4 weeks post-infection; 8 animals from each *Helicobacter*-infected group and 7 animals from the control group at 9 weeks post-infection) were euthanized by cervical dislocation under deep isoflurane anaesthesia (5%). The stomach from each mouse was resected and samples were taken for histopathological examination and quantitative RT-PCR analysis.

Histopathology and quantification of colonizing *Helicobacter* spp.

A longitudinal section, starting from the end of the forestomach and comprising the antrum and the fundus of the stomach and part of the duodenum, was fixed in 10% phosphate buffered formalin and embedded in paraffin for histopathological examination as previously described (Liu et al., 2014). A haematoxylin/eosin (H&E) staining was performed on a paraffin slide of 5µm to score the intensity of the gastritis according to the Updated Sydney System, but with some modifications (Liu et al., 2014; Flahou et al., 2010). DNA from the antrum and fundus of the stomach was extracted (Joosten et al., 2013) and the number of colonizing *H. heilmannii* ASB1wt, ASB1*ΔhofE*, ASB1*ΔhofF* and ASB1*ΔhofG* and *H. pylori* SS1 and STM138*ΔhofF* per mg gastric tissue (antrum and fundus of the stomach) was determined in the DNA samples using *H. heilmannii*- and *H. pylori* specific qPCRs based on a short fragment of the *ureAB* gene cluster (Liu et al., 2014; Joosten et al., 2013). For

quantification of *H. heilmannii* and *H. pylori* DNA in the tissue samples, the Bio-Rad CFX Manager (version 1.6) software was used.

Quantification of host gene expression levels.

Total RNA extracted from the stomach samples was utilized for first strand cDNA synthesis using iScript™ cDNA Synthesis Kit (Bio-Rad) (Liu et al., 2014). Quantitative RT-PCR was carried out for measuring gene expression levels of murine gastric mucins (Muc1, Muc5AC, Muc6 and Muc13), the human IL-8 related mouse proteins (Mip-2, Kc and Lix), IL-1β and TNF-α. The murine housekeeping genes *PPIa, H2afz and HPRT* were included as reference genes. Primer sequences are shown in Table S3. The quantitative PCR reactions were performed as described before (Liu et al., 2014). No-template-control reaction mixtures were included and all samples were run in duplicate. The Ct-values were normalized to the geometric mean of the Ct-values from the housekeeping genes. Results are shown as fold changes of mRNA expression in infected animals relative to mRNA expression levels in control animals. Fold changes were calculated using ΔΔCT method (Livak et al., 2001) with mean of Ct-values from 5 or 7 uninfected mice as controls.

Statistical analysis.

Statistical analysis was performed using SPSS Statistics 21 software (IBM) package. Gastritis scores, the number of colonizing bacteria in the murine stomach and cytokine production in the cell culture supernatant were analyzed using the non-parametric Mann-Whitney *U* test to compare groups. Bacterial and host gene expression was compared between different *in vitro* conditions or infected groups and controls using a Bonferoni post hoc test (ANOVA). Differences were considered statistically significant at $p \leq 0.05$.

Results

The effect of pH, temperature, H_2O_2, gastric mucins and gastric epithelial cells on the expression of the *H. heilmannii hof* genes located in a 10kb locus.

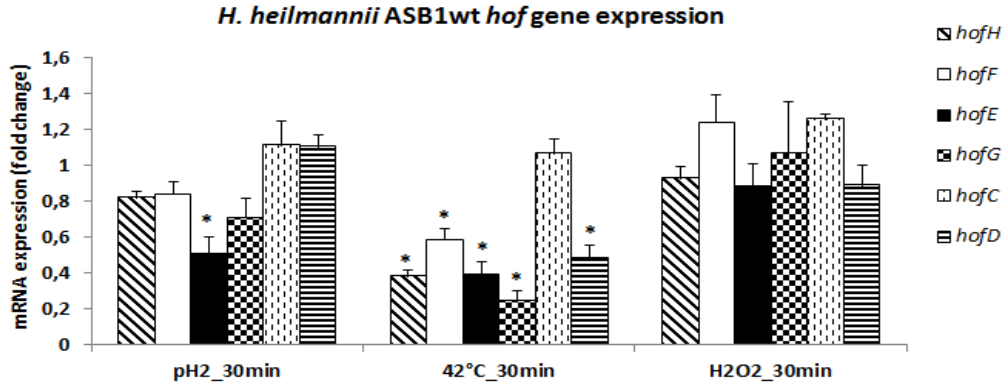
As the pH in the stomach varies from pH2 in the gastric lumen to pH7 at surface epithelium and in the glands, we hypothesized that the effect of pH can modulate expression of *H. heilmannii omp* genes involved in gastric colonization. At pH2, mRNA expression of only *hofE* was decreased after 30 min and 1h of incubation (p=0.002; Figure 2A & Table S1). Interestingly, the mRNA expression levels of *hofE*, *hofF* and *hofG* were increased after 24h

growth at pH7 ($p<0.05$; Fig. 2B & Table S1).

The effect of exposure to 42°C (a gastric environmental stress condition) for 30 min and 1h on the expression of the *hof* genes, showed that all genes, with the exception of *hofC*, were downregulated ($p<0.05$; Figure 2A & Table S1). On the contrary, exposure of *H. heilmannii* to H_2O_2 to mimic oxidative stress did not induce statistically significant changes in expression of the *hof* genes (Figure 2A & Table S1).

It has been shown that mucins can alter the expression of genes involved in binding of *H. pylori* to the gastric mucosa (Skoog et al., 2012). Incubation of *H. heilmannii* with mucins resulted in an increase ($p<0.01$) in mRNA expression of *hofC*, *hofD*, *hofE*, *hofF* and *hofG* as shown in Figure 2B and Table S1. Subsequently, *H. heilmannii hof* gene expression was also studied during co-incubation of this bacterium with human gastric epithelial cells. Adhesion to MKN7 cells did not cause statistically significant changes in the expression of *H. heilmannii hofC*, *hofD*, *hofE*, *hofF*, *hofG* and *hofH* (Figure 2B & Table S1).

2A



2B

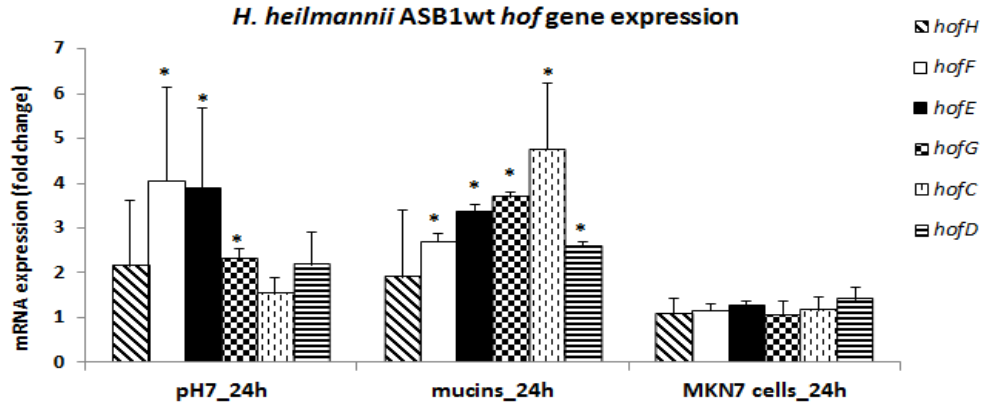


Figure 2: *H. heilmannii hof* gene expression in response to exposure at pH2, 42°C and H_2O_2 for 30 min (A) and pH7, a gastric mucin sample at pH2 (Human 2 obtained from a gastric tumor and on which H. heilmannii binds to) and MKN7 cells at pH7 (B) for 24h (B). Each experiment was repeated at least 3 times. Expression levels of *hofH*, *hofF*, *hofE*, *hofG*, *hofC* and *hofD* from the ASB1 wildtype (wt) strain are shown. Data are presented as fold change in gene expression normalized to two reference genes (*atpA* and *efp* genes for *H. heilmannii*) and relative to the results for *H. heilmannii* grown under normal *in vitro* conditions (pH5) or in the absence of mucins and cells (3). Data are shown as means + standard deviation. Significant differences in expression level between *H. heilmannii* grown under the different environmental conditions (acid, temperature, H_2O_2, mucins and cells) and normal *in vitro H. heilmannii* growth (pH5) or *H. heilmannii* grown without mucins or cells are indicated by * $p<0.05$ (ANOVA).

A clear reduction in binding of *H. heilmannii ΔhofF, ΔhofE* and *ΔhofG* mutants to human gastric mucins and epithelial cells.

Genomic analysis of the *H. heilmannii* ASB1wt *hof* gene locus revealed that only HofE, HofF and HofG had a predicted signal sequence (Figure 1). Based on this finding and the results of the above *hof* gene expression analyses, the genes encoding HofE, HofF and HofG were selected for further analyses. *H. heilmannii* strains lacking HofE, HofF or HofG were developed by chromosomal insertional mutagenesis. The mRNA expression pattern of the other *hof* genes residing in the *hof* locus of the *H. heilmannii* ASB1*ΔhofE,* ASB1*ΔhofF* and ASB1*ΔhofG* mutants was also examined under normal growth (pH5), different stress conditions and in co-culture with gastric mucins and epithelial cells. Results are shown in Table S1.

Under normal growth conditions (pH5 for 24h), only the expression of *hofE* in the *ΔhofF* mutant was decreased (p=0.001). At pH7, the expression pattern of most of the *hof* genes in the different mutant strains was comparable to their expression in the wildtype strain. Only the *hofH* gene was overexpressed (p=0.002) in ASB1*ΔhofE* whereas expression of *hofG* did not reach statistical significance in ASB1*ΔhofF* (Table S1).

However, under acid, high temperature and oxidative stress, the *hofE* gene in ASB1*ΔhofF* and the *hofF* gene in ASB1*ΔhofE* were increased (p=0.001 for *hofE* and *hofF*; Table S1). Additionally, exposure at 42°C also changed the expression pattern of *hofH* and *hofG* in the mutants (p=0.001 for *hofH*; p=0.003 for *hofG*) compared to their expression pattern in the wildtype strain (Table S1).

In response to gastric mucins, expression of the *hof* genes in the mutant lacking HofE did not reach statistical significance, whereas only *hofG* was upregulated in the ASB1*ΔhofF* mutant (p=0.001; Table S1). Co-culture of ASB1*ΔhofE*, ASB1*ΔhofF* and ASB1*ΔhofG* with MKN-7

cells showed a similar *hof* gene expression pattern in the different mutants compared to its expression pattern in ASB1wt (Table S1).

Subsequently, the binding ability of the *H. heilmannii* ASB1*ΔhofE*, ASB1*ΔhofF* and ASB1*ΔhofG* mutants to gastric mucins and epithelial MKN7 and GSM06 cells was investigated. Mucin binding was investigated at pH2 and pH5, with pH2 representing the environment present at the luminal side of the mucus layer, and pH5 the environment closer to the cell surface. A weak binding to the gastric mucin samples was found for the *H. heilmannii* ASB1wt strain at pH2 and pH5 (Figures 3A & 3B). The level of binding at pH2 was lower with the ASB1*ΔhofE*, ASB1*ΔhofF* and ASB1*ΔhofG* mutants compared to the wildtype strain, and a similar trend was present at pH5 ($p<0.05$, Figures 3A & 3B).

H. heilmannii ASB1wt strongly binds to human MKN7 cells (Figures 3C & 3D) and murine GSM06 cells (Figures 3C & 3H). A lower number of ASB1*ΔhofE*, ASB1*ΔhofF* and ASB1*ΔhofG* mutant bacteria bound to the human-derived MKN7 cells and mouse-derived GSM06 cells than the *H. heilmannii* ASB1wt strain at pH7 ($p<0.05$; Figures 3C to 3K). Reduction in binding was more pronounced for the ASB1*ΔhofE* mutant (Figure 3C).

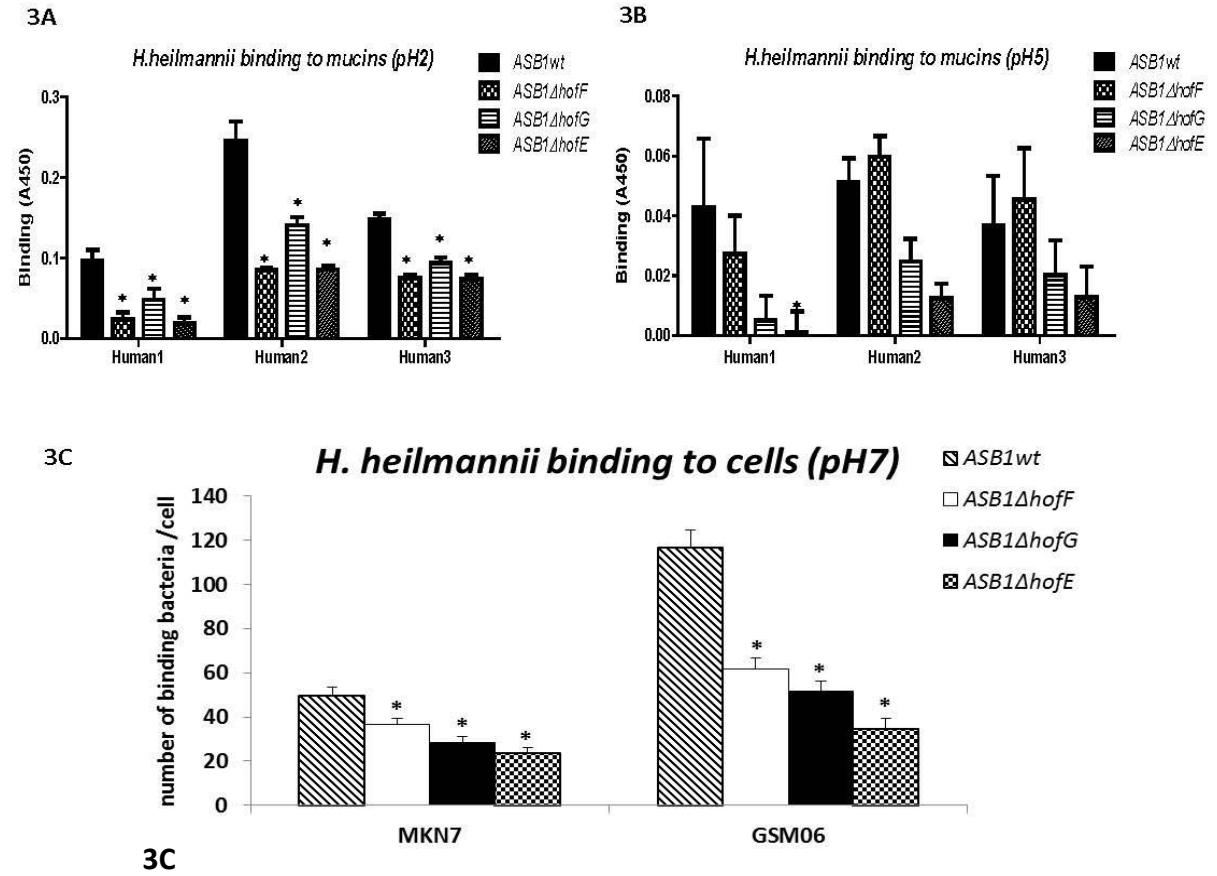


Figure 3: *In vitro* binding capacity of *H. heilmannii* and its *Δhof* mutants to gastric mucins and mucus-secreting epithelial cells.

In vitro binding of ASB1wt, ASB1*ΔhofE,* ASB1*ΔhofF* and ASB1*ΔhofG* to different human gastric mucin samples (Human 1 obtained from a healthy stomach; Human 2 and human 3 obtained from a gastric tumor) at pH2 (A) and pH5 (B). Binding was quantified by measuring the OD at 450 nm. Subsequently, scanning electron microscopy was performed for visualization of binding at pH 7 of *H. heilmannii* ASB1wt (D), ASB1*ΔhofF* (E), ASB1*ΔhoGF* (F) and ASB1*ΔhofE* (G) to MKN7 cells and ASB1wt (H), ASB1*ΔhofF* (I), ASB1*ΔhofG* (J) and

ASB1*ΔhofE* (K) to GSM06 cells. Bars: 10µm. The mean number of *H. heilmannii* and its mutants binding MKN7 cells and GSM06 cells at pH7 was calculated (C). Significant differences in binding between the wildtype strain and a mutant strain are indicated by * $p<0.05$ (Mann-Withney U test).

H. heilmannii HofE and HofF and *H. pylori* HofF play a role in MUC13 expression in human gastric epithelial cells.

We recently suggested a potential role for Muc13 in gastric *Helicobacter* colonization in mice (Liu et al., 2014). Therefore, human MUC13 mRNA expression was examined after co-culture of *H. heilmannii* and gastric epithelial MKN7 cells for 24h. The *H. pylori* P1 strain was included for comparison. For both *Helicobacter* species, mRNA expression of MUC13 was increased (Fold change for ASB1wt 7.66±2.51 ($p=0.003$) and for P1 4.21±0.39 ($p=0.001$); Figure 4A). To investigate the role of the *H. heilmannii* HofE, HofF and HofG proteins in MUC13 expression, MKN7 cells were also incubated with the ASB1*ΔhofE*, ASB1*ΔhofF* and ASB1*ΔhofG* mutant strains. The *H. pylori* STM138*ΔhofF* strain was included for comparison. Only ASB1*ΔhofE*, ASB1*ΔhofF* and STM138*ΔhofF* were unable to increase MUC13 expression significantly. The other strains induced an upregulation of MUC13 expression ($p=0.003$ for ASB1wt; $p=0.004$ for P1; $p=0.013$ for ASB1Δ*hofG*; Figure 4A).

IL-1β is associated with increased MUC13 expression in human gastric epithelial cells.

In the stomach, the activation of mucin expression can be regulated by inflammatory cytokines induced in response to *Helicobacter* colonization of the gastric mucosa (Mejias-Luque et al., 2008). Therefore, the supernatant of *Helicobacter*-infected and uninfected MKN7 cells was screened for cytokine production (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17α, IFNγ, INFα, GM-CSF) using ELISA assays. Only an increased IL-8 production was detected in the supernatant of cells infected with *H. heilmannii* and *H. pylori* wildtypes and their mutant strains (Figure 4B; $p<0.05$) and no difference in IL-8 production was seen between *Helicobacter* wildtype- and mutant-infected cells (data not shown). To identify whether this pro-inflammatory cytokine was involved in the activation of MUC13 expression, uninfected MKN7 cells were treated with human recombinant IL-8. MUC13 expression was not induced by IL-8. Subsequently, uninfected MKN-7 cells were treated with other inflammatory cytokines, including IL-1β, IL-6 and TNF-α. Interestingly, IL-1β was able to increase MUC13 significantly ($p=0.025$; Figure 4C). Additionally, IL-8 mRNA expression was also analyzed in the cells after cytokine treatment. Expression of this cytokine was induced by IL-1β and TNF-α ($p=0.001$ for IL-1β; $p=0.012$ for TNF-α; Figure 4D).

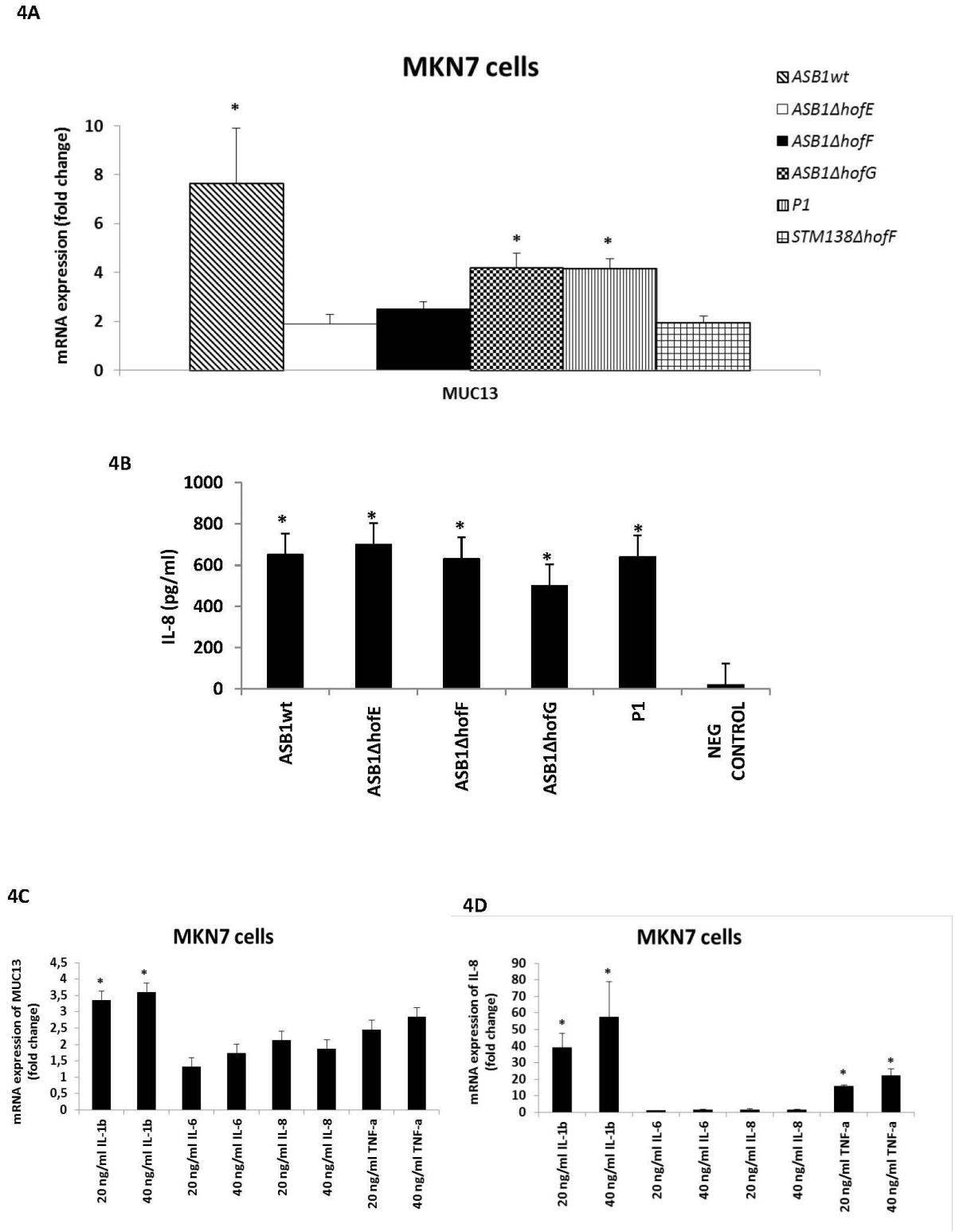


Figure 4: Human MUC13 expression during incubation of *H. heilmannii* ASB1wt ASB1*ΔhofF*, ASB1*ΔhofE* and ASB1*ΔhofG* and *H. pylori* P1 and STM138*ΔhofF* to gastric epithelial cells for 24h (A).

Data are presented as fold change in gene expression normalized to 1 reference gene and relative to the results for uninfected cells. Data are shown as means + standard deviation. Significant differences in expression level between *Helicobacter*-infected gastric cells and uninfected gastric cells are indicated by * $p<0.05$ (ANOVA). (B)

Presence of Human IL-8 in cell culture media measured at OD_{450nm} at 24h after inoculation with ASB1wt, ASB1*ΔhofF*, ASB1*ΔhofE*, ASB1*ΔhofG*, P1 and STM138*ΔhofF*. The concentration of IL-8 (pg/ml) was calculated using a standard curve. Significant difference between infected and uninfected cells are indicated by * $p<0.05$ (ANOVA). Human MUC13 (C) and IL-8 (D) expression measured after treatment with IL-1β, IL-6, IL-8 or TNF-α (20 ng/ml or 40 ng/ml) for 24h. Significant difference between cytokine-treated and untreated cells are indicated by * $p<0.05$ (ANOVA).

Significantly reduced induction of inflammation and colonization by *H. heilmannii ΔhofF* and *ΔhofE* and *H. pylori ΔhofF* in the stomach of mice.

To confirm the role of HofE, HofF and HofG in *H. heilmannii* colonization, the ASB1wt, ASB1*ΔhofE*, ASB1*ΔhofF* and ASB1*ΔhofG* strains were studied in a mouse model. The rodent-adapted *H. pylori* SS1 and STM138*ΔhofF* strains were included for comparison. Inflammation in the stomach of all mice was scored. For all control animals, the histomorphology was considered to be normal, with only minor inflammatory cell infiltration in the gastric mucosa. The results of the mice at 9 weeks post-infection are shown in Figure 5A. A statistically significant difference ($p<0.05$) was demonstrated between the inflammation scores of ASB1-infected mice on the one hand and ASB1*ΔhofE*- or ASB1*ΔhofF*-infected mice on the other hand. A similar difference was seen between mice infected with SS1 or STM138*ΔhofF*. On the contrary, no difference in inflammation was seen between ASB1- and ASB1*ΔhofG*-infected mice (Figure 5A). The histomorphology in the stomach of the ASB1*ΔhofE*-, ASB1*ΔhofF*- and STM138*ΔhofF*-infected mice was comparable to that of the control animals (Figures 5C, 5D & 5G). Inflammation in ASB1-, ASB1*ΔhofG*- and SS1-infected mice was characterized by mononuclear and polymorphonuclear cell infiltration in the lamina propria mucosae, the tunica submucosa or both, depending on the individual animal (Figures 5B, 5E & 5F). Throughout the experiment, all control animals were negative for *Helicobacter* DNA in quantitative RT-PCR assays. At 4 and 9 weeks post-infection, *Helicobacter* DNA was found in both the antrum and the fundus of the stomach from all infected animals with a higher amount seen in the antrum (Figures 6A & 6B). The ASB1*ΔhofE*, ASB1*ΔhofF* and STM138*ΔhofF* mutants showed a clear and lower colonization capacity compared to the *H. heilmannii* and *H. pylori* wildtype strains ($p=0.002$ for ASB1*ΔhofE* and ASB1*ΔhofF*; $p=0.004$ for STM138*ΔhofF*; Figures 6A & 6B). The ASB1*ΔhofG* mutant colonized the stomach at an equal capacity as the ASB1wt strain (Figure 6). In general, *H. heilmannii* colonized the antrum of the stomach at a higher level than *H. pylori* ($p=0.002$; Figure 6A).

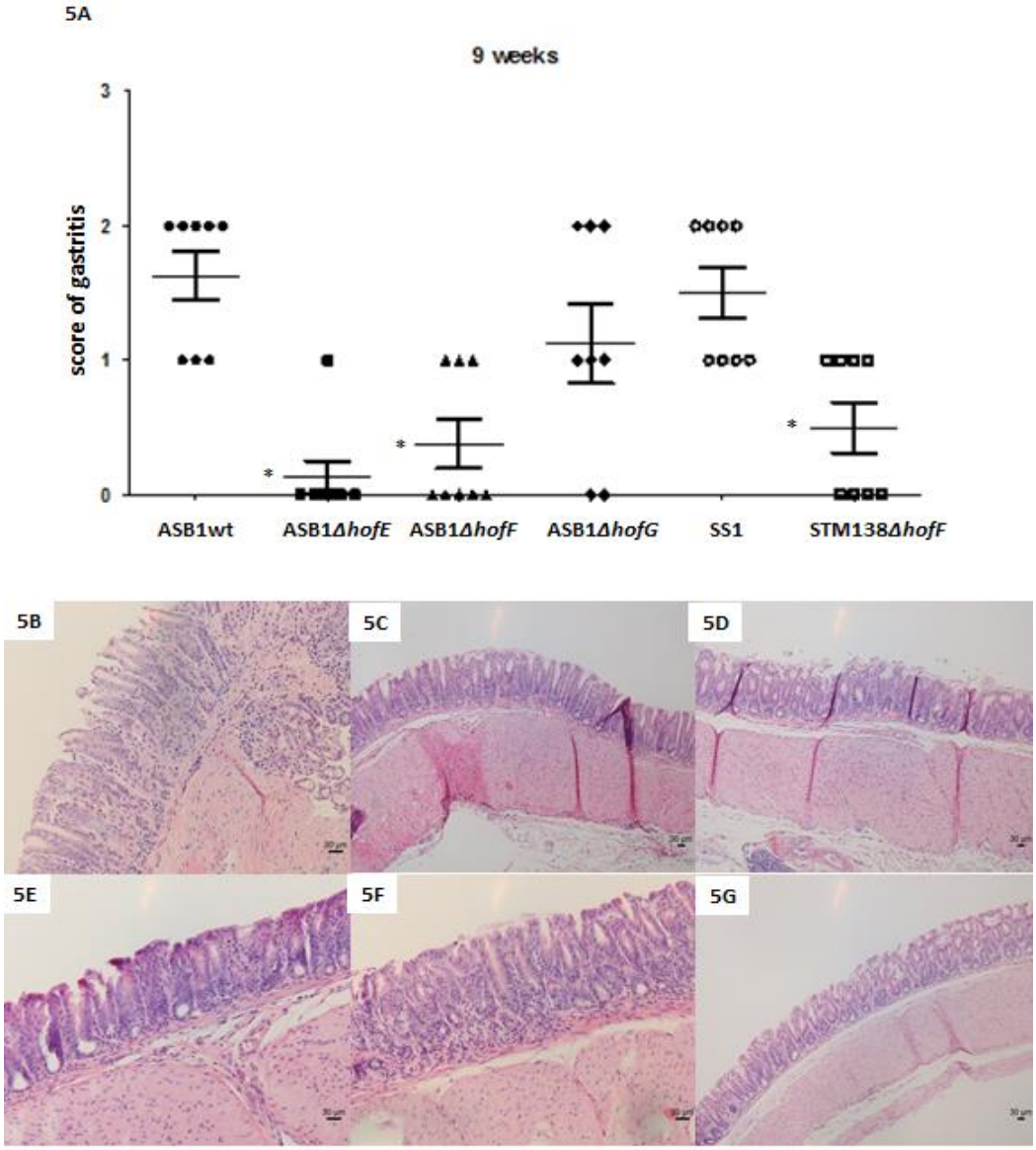
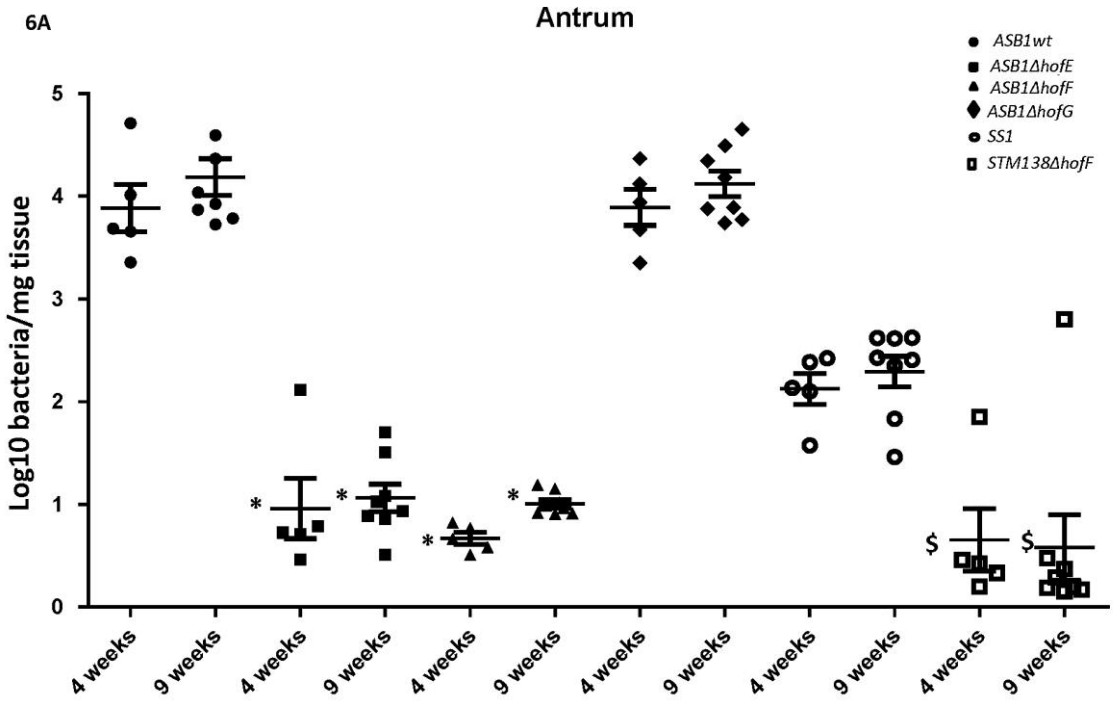


Figure 5: Gastric inflammation in *H. heilmannii* ASB1wt-, ASB1*ΔhofF*-, ASB1*ΔhofE*-, ASB1*ΔhofG*-, and *H. pylori* P1- and STM138*ΔhofF*-infected Balb/c mice. (A) Gastric inflammation was scored on a scale of 0 to 4 (0: no infiltration with mononuclear and/or polymorphonuclear cells; 1: mild diffuse infiltration with mononuclear and/or polymorphonuclear cells or the presence of one small (50–200 cells) aggregate of inflammatory cells; 2: moderate diffuse infiltration with mononuclear and/or polymorphonuclear cells and/or the presence of 2–4 inflammatory aggregates; 3: marked diffuse infiltration with mononuclear and/or polymorphonuclear cells and/or the presence of at least five inflammatory aggregates; 4: diffuse infiltration of large regions with large aggregates of mononuclear and/or polymorphonuclear cells). Individual animals are depicted as symbols around the mean (lines). (B-G) H&E staining of the antrum of the stomach from a *Helicobacter*-infected Balb/c mouse. Inflammation in ASB1- (B), ASB1*ΔhofG*- (E) and SS1(F)-infected mice was characterized by mononuclear and polymorphonuclear cell infiltration in the lamina propria mucosae, the tunica submucosa or both. The histomorphology in the stomach of the ASB1*ΔhofE*- (C), ASB1*ΔhofF*- (D) and STM138*ΔhofF*(G)-infected mice was comparable to the control animals showing only minor inflammatory cell infiltration in the gastric mucosa. Bar = 30 µm.

6A


Antrum
ASB1wt
ASB1ΔhofE
ASB1ΔhofF
ASB1ΔhofG
SS1
STM138ΔhofF
Log10 bacteria/mg tissue
0
1
2
3
4
5
*
$
4 weeks
9 weeks

6B

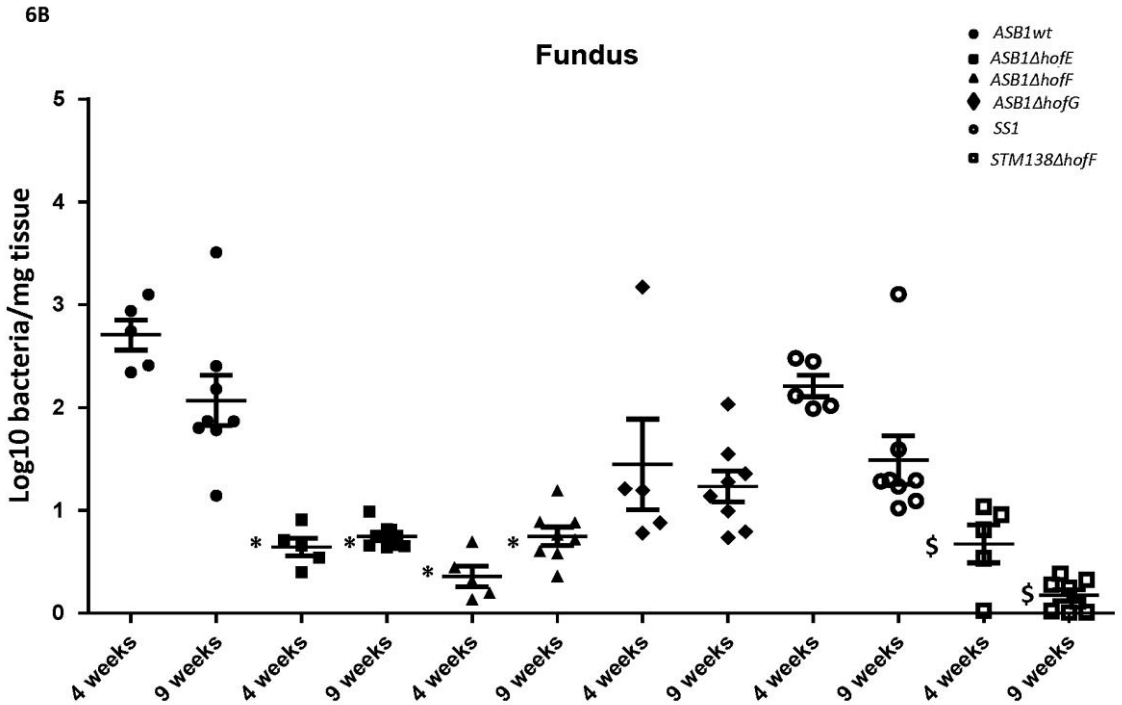
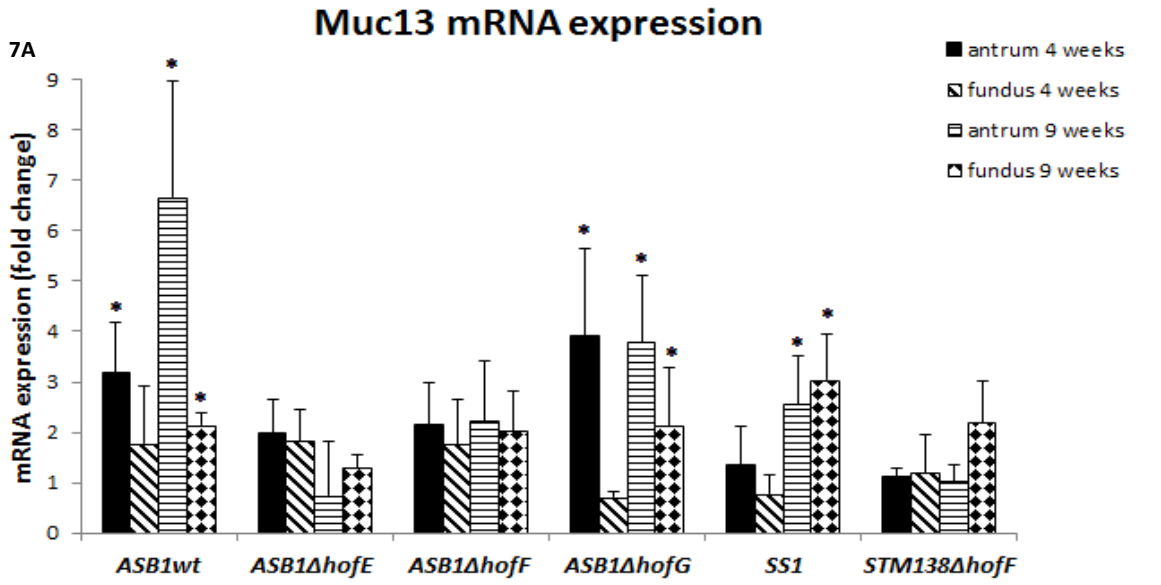

Fundus
ASB1wt
ASB1ΔhofE
ASB1ΔhofF
ASB1ΔhofG
SS1
STM138ΔhofF
Log10 bacteria/mg tissue
0
1
2
3
4
5
*
$
4 weeks
9 weeks

Figure 6: Colonization capacity of *H. heilmannii* ASB1*wt*, ASB1*ΔhofF,* ASB1*ΔhofE,* ASB1*ΔhofG* and *H. pylori* SS1 and STM138*ΔhofF* after experimental infection. Colonization capacity is shown as $\log_{10}$ values of bacteria per mg tissue, detected with quantitative RT-PCR in the antrum (A) and the fundus (B) of the stomach. Animals in which no *Helicobacter* DNA could be detected in the stomach were set as 0. Individual animals are depicted as figures around the mean (lines). Statistical significant differences between animals infected with *Helicobacter* and control animals are indicated by * (*H. heilmannii*) or $ (*H. pylori*) (Mann-Whitney *U* test, $p < 0.05$).

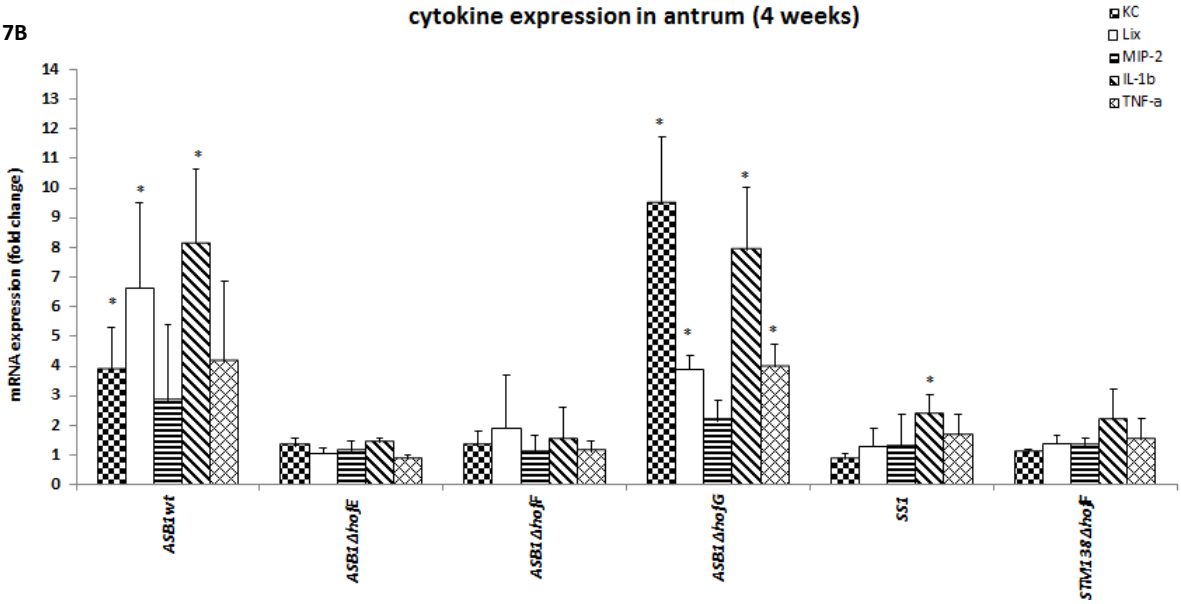
The *H. heilmannii* HofE and HofF and *H. pylori* HofF proteins play a role in IL-1β-induced Muc13 expression in the stomach of mice.

Changes in mRNA expression of mucins in the stomach of control and *Helicobacter*-infected mice were investigated. No changes in Muc1 expression were seen in the stomach during the whole experiment (Table S2). mRNA expression of Muc5AC was decreased ($p<0.05$) in the antrum of the stomach of SS1- and STM138*ΔhofF*-infected mice at 9 weeks post-infection (Table S2). Muc6 was upregulated at 9 weeks post-infection in the fundus of all infected animals compared to the uninfected controls ($p<0.05$; Table S2). The mRNA expression of Muc13 was increased at 4 weeks post-infection in the antrum of ASB1- and ASB1*ΔhofG*-infected mice ($p<0.05$; Figure 7A, Table S2) and at 9 weeks post-infection in both the antrum and fundus of ASB1-, ASB1*ΔhofG*-, and SS1-infected mice ($p<0.05$; Figure 7A, Table S2). Interestingly, no significant change in Muc13 expression was seen in the stomach of ASB1*ΔhofE*-, ASB1*ΔhofF*- and STM138*ΔhofF*-infected mice during the whole experiment at either time point (Figure 7A, Table S2). Gene expression of Mip-2, Kc and Lix, the human IL-8 related mouse proteins, was increased in the stomach of most *Helicobacter*-infected groups at 4 and 9 weeks post-infection ($p<0.05$; Figures 7B to 7E). Upregulation of Mip-2 was restricted to the fundus ($p<0.05$; Figures 7C &,7E, Table S2) whereas expression of Kc and Lix was induced in both the antrum and fundus of the stomach ($p<0.05$; Figures 7B to 7E, Table S2). Also the ASB1*ΔhofE* mutant stimulated Mip-2, Kc and Lix expression, although only in the fundus of the stomach ($p<0.05$; Figures 7C & 7E, Table S2). The mRNA expression of the pro-inflammatory cytokines IL-1β and TNF-α was significantly increased ($p<0.05$) in the antrum of the stomach of ASB1wt-, ASB1*ΔhofG*- and SS1-infected mice at 4 and 9 weeks post-infection (Figures 7B & 7D, Table S2).

7A


Muc13 mRNA expression
mRNA expression (fold change)
antrum 4 weeks
fundus 4 weeks
antrum 9 weeks
fundus 9 weeks
ASB1wt
ASB1ΔhofE
ASB1ΔhofF
ASB1ΔhofG
SS1
STM138ΔhofF

7B


cytokine expression in antrum (4 weeks)
mRNA expression (fold change)
KC
Lix
MIP-2
IL-1b
TNF-a
ASB1wt
ASB1ΔhofE
ASB1ΔhofF
ASB1ΔhofG
SS1
STM138ΔhofF

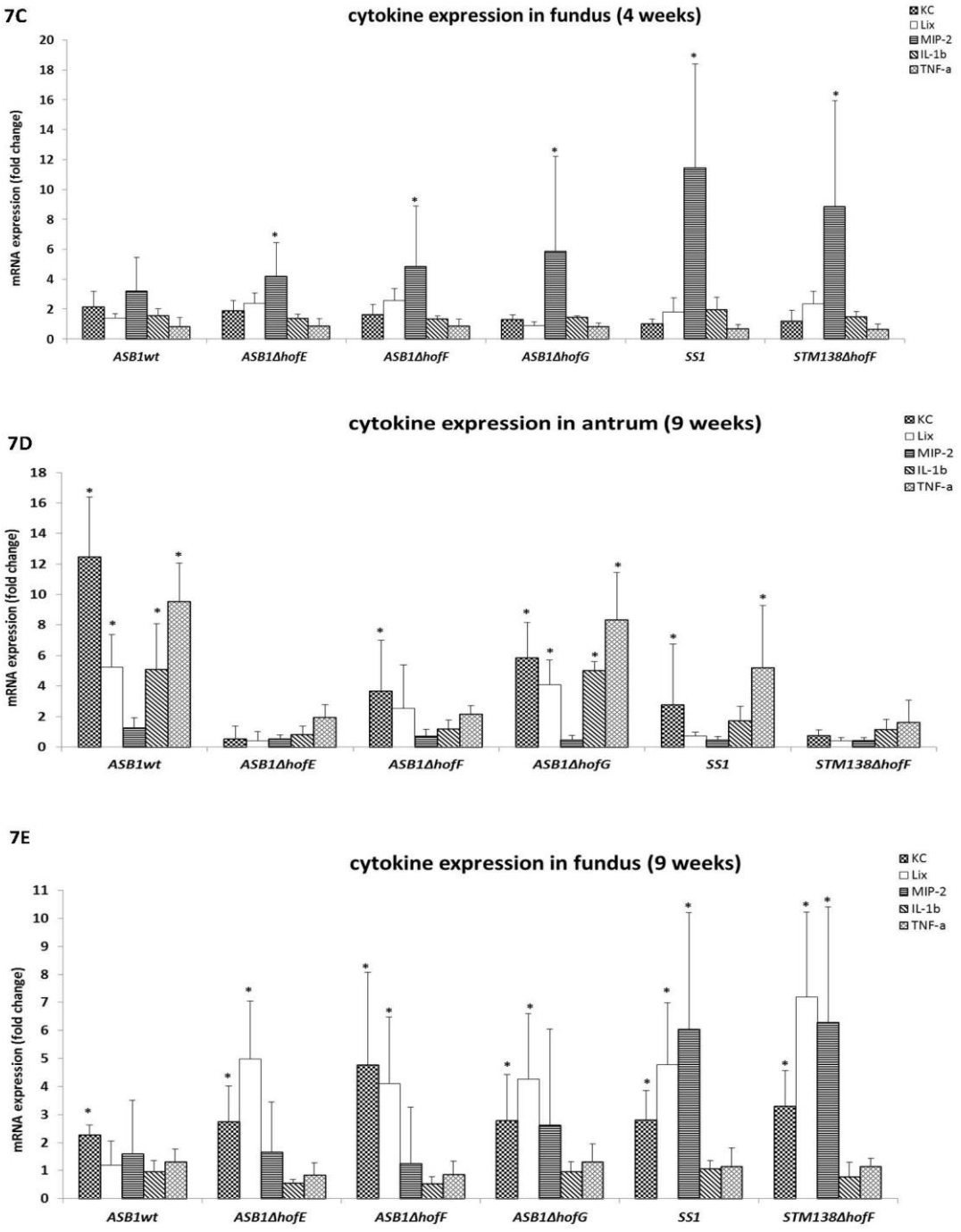


Figure 7: Expression of Muc13 (A), the human IL-8 related mouse genes (*Mip-2*, *Kc* and *Lix*), IL-1β, and TNFα in the stomach of *H. heilmannii* ASB1wt-, ASB1*ΔhofF*-, ASB1*ΔhofE*-, ASB1*ΔhofG*-, and *H. pylori* P1- and STM138*ΔhofF*-infected and control Balb/c mice. Expression in the antrum (4 weeks A&B; 9 weeks (A&C)) and fundus (4 weeks (A&D); 9 weeks (A&E)) of the stomach at 4 and 9 weeks post-infection is shown. Data are

presented as fold change in gene expression normalized to 3 reference genes and relative to the results for the negative-control group. Data are shown as means + standard deviation. Significant differences in expression level between the infected groups and the negative-control group at a certain time-point are indicated by * $p<0.05$ (ANOVA).

Discussion

Upon exposure to low pH and high temperature, expression of most of the *H. heilmannii* genes residing in the *hof* locus and in particular *hofE*, was decreased. The corresponding Hof OMPs contain predicted transmembrane beta-barrrel sheets which can act as diffusion channels that allow small molecules to pass across the outer membrane (Smet et al., 2014). This reduced expression of the *H. heilmannii hof* genes might thus lead to a decrease in the permeability of the outer membrane and in this way limit the accessibility of damaging agents to the periplasm and beyond. Similar findings have been described for several *H. pylori* Hop proteins (de Vries et al., 2001; Huang et al., 2011; Wen et al., 2003; Merell et al., 2003; Shan et al., 2015; Giannakis et al., 2009).

Adherence to the gastric mucosa is assumed to play an important role in the initial colonization of gastric helicobacters (McGuckin et al., 2011) and it can be expected that this event is associated with increased expression of genes playing a role in adhesion. However, in the present study, expression of none of the *hof* genes was changed during adhesion of *H. heilmannii* to human gastric epithelial MKN7 cells. On the other hand, contact of the bacterium with human gastric mucins resulted in an upregulation of almost all *hof* genes examined and exposure to pH7 significantly increased the expression of *hofE*, *hofF* and *hofG*. This may suggest that *H. heilmannii* modifies the expression of its *hof* genes upon contact with mucins and as a reaction to changing pH conditions when it moves away from the acidic lumen of the stomach towards the gastric mucosa. The enhanced expression of these genes at pH7, which resembles the pH close to the gastric epithelium (Skoog et al., 2012), may indicate that the HofE, HofF and HofG OMPs play a role in adhesion of *H. heilmannii* to gastric epithelial cells. These are also the only Hof proteins, encoded by the *H. heilmannii hof* gene locus, that carry a predicted known N-terminal signal sequence, which may promote their translocation into the outer membrane after enhanced synthesis (Odenbreit et al., 1999; Carlsson et al., 2006). Therefore, *ΔhofE*, *ΔhofF* and *ΔhofG* mutants were created for further investigating the role of HofE, HofF and HofG in adhesion and colonisation.

A complementation test to verify the phenotype of the mutants as frequently used in *H. pylori* research, is currently unavailable for *H. heilmannii* due to its very fastidious nature. Further

studies, including the identification of a suitable locus for the integration of the target gene, the selection of a suitable promoter and the evaluation of several marker genes (i.e: chloramphenicol, erythromycin and kanamycin resistant cassettes), are essential to set up such an assay.

The *hof* gene expression pattern in the created mutants was first analysed to verify if the lack of a certain *hof* gene had an impact on the expression of other *hof* genes in that same locus. Under normal growth conditions, only the expression of *hofE* in the *ΔhofF* mutant was decreased, indicating that the presence of the *hofF* gene is essential for a normal *hofE* expression. Protein analyses are necessary to examine whether translation of *hofE* mRNA in this mutant results in a functional HofE protein. Compared to the *H. heilmannii* wildtype strain, exposure of these mutants to different stress conditions and mucins induced a change in the expression of the other *hof* genes in the *ΔhofF* and *ΔhofE* mutants suggesting that disruption of *hofE* and *hofF* may have an impact on the expression of the other genes in the *hof* locus.

In general, binding to the gastric mucin samples was rather low for the *H. heilmannii* wildtype strain. Mucins can carry in the order of 100 different carbohydrate structures, which provide the mucins with a bottle-brush appearance and make them act as receptors for microorganisms (McGuckin et al., 2011). The absence of carbohydrates having a strong binding ability with *H. heilmannii* in the used mucin samples, might explain the low binding capacity of this pathogen.

The ability to bind to mucins was, however, even lower for the *H. heilmannii ΔhofE*, *ΔhofF* and *ΔhofG* mutant strains compared to the wildtype strain. Similarly, the capacity to adhere to gastric epithelial cells was also lower for these mutant strains, compared to the wildtype strain. These results confirm that HofE, HofF and HofG are involved in the attachment of *H. heilmannii* to gastric mucins and epithelial cells.

The role of these Hof proteins in colonization of the gastric mucosa by *H. heilmannii* was further investigated *in vivo*. The well-studied mouse-adapted *H. pylori* SS1 strain (Liu et al., 2014; Crabtree et al., 2002) and a *H. pylori ΔhofF* mutant (Kavermann et al., 2003) were included for comparison. The *H. heilmannii ΔhofE* and *ΔhofF* mutants colonized the stomach, in particular the antrum of the stomach, at a clearly lower level than the *H. heilmannii* wildtype strain. The previously shown role of *H. pylori* HofF in gastric colonization was also confirmed (Kavermann et al., 2003). These findings, together with the results of the cell binding experiments described above, demonstrate that both *H. heilmannii* HofE and HofF are

essential in gastric *Helicobacter* colonization, in particular adherence to the gastric mucosa. It remains to be elucidated whether deletion of both *hofE* and *hofF* would result in a complete loss of adhesion of the *H. heilmannii ΔhofFΔhofE* mutant to the gastric mucosa. A similar phenomenon has been described for the *alpA* and *alpB* genes in *H. pylori* (Odenbreit et al., 1999; Senkovich et al., 2011). These genes, both encoding OMP Hop adhesins, are located closely together in a locus. Deletion of this gene locus in *H. pylori* resulted in a complete loss of binding to human gastric tissue sections (Oleastro and Menard, 2013; Senkovich et al., 2011).

On the contrary, the *H. heilmannii ΔhofG* mutant colonized the murine stomach at a comparable level as the *H. heilmannii* wildtype strain. Although our *in vitro* studies showed that HofG plays a role in adherence to human gastric epithelial MKN7 cells, this OMP is apparently not an essential colonization factor in our mouse model. It remains to be determined whether it is involved in gastric colonization in human patients.

In the present study, the pathway regulating gastric MUC13 expression as a response to *Helicobacter* colonization was further investigated. A schematic representation of the presumptive cascade of events involved in gastric MUC13 expression that occur during adhesion of *H. heilmannii* to epithelial cells is shown in Figure 8. An increased expression of MUC13 in response to colonization of *H. heilmannii* and *H. pylori* to human gastric epithelial cells was shown. This finding confirms the recently suggested role of murine Muc13 in gastric *Helicobacter* colonization (Liu et al., 2014). However, it remains to be further elucidated whether attachment of these helicobacters to the cell surface is essential for the induction of MUC13 expression. How human MUC13 and murine Muc13 influence the *Helicobacter* colonization process *in vivo* has not yet been unraveled. Analyses of the stomach of *Helicobacter*-infected Muc13$^{-/-}$ mice might shed more light on this.

The activation of gastric MUC13 expression can be regulated by inflammatory cytokines induced in response to *Helicobacter* colonization of the gastric mucosa as has been described for other membrane-associated mucins (Mejias-Luque et al., 2008). In this study, an increased IL-8 production was found in the supernatant of human gastric epithelial cells infected with *H. heilmannii* or *H. pylori*. Therefore, the role of IL-8 as well as other pro-inflammatory cytokines in MUC13 expression was further investigated *in vitro*. Interestingly, MUC13 expression was induced by IL-1β (Figure 8) and not by IL-8. IL-1β also caused increased IL-8 mRNA expression in these human gastric epithelial cells (Figure 8). Previous *in vivo* experiments showed that mRNA levels of IL-1β were clearly upregulated in the stomach of

rodents suffering from gastritis induced by *H. heilmannii* and *H. pylori* (Joosten et al., 2013; unpublished data). Also in our mice experiment, IL-1β expression was significantly increased in the antrum of the stomach of *H. heilmannii* ASB1wt- and *H. pylori* SS1-infected animals. These findings suggest that IL-1β is induced in the stomach as a response to *Helicobacter* colonization and on its turn plays a role in the expression of MUC13 and IL-8 in the gastric epithelium. The signal transducers regulating the IL-1β-induced MUC13 and IL-8 expression pathway in the stomach have not yet been identified (Figure 8). IL-1β plays a crucial role in the development of gastric pathologies (Joosten et al., 2013). To be active, pro-IL-1β must be cleaved by the inflammasome, an intracellular multiprotein oligomer implicated in the activation of inflammatory processes (Semper et al., 2014; Figure 8). It has recently been shown that *H. pylori*-induced IL-1β secretion is mediated by the nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome which requires the *H. pylori* vacuolating cytotoxin A (VacA) and cag pathogenicity island (Semper et al., 2014). *H. heilmannii* lacks both VacA and cagPAI (Liu et al., 2014). How the inflammasome is activated by *H. heilmannii* is so far unknown (Figure 8).

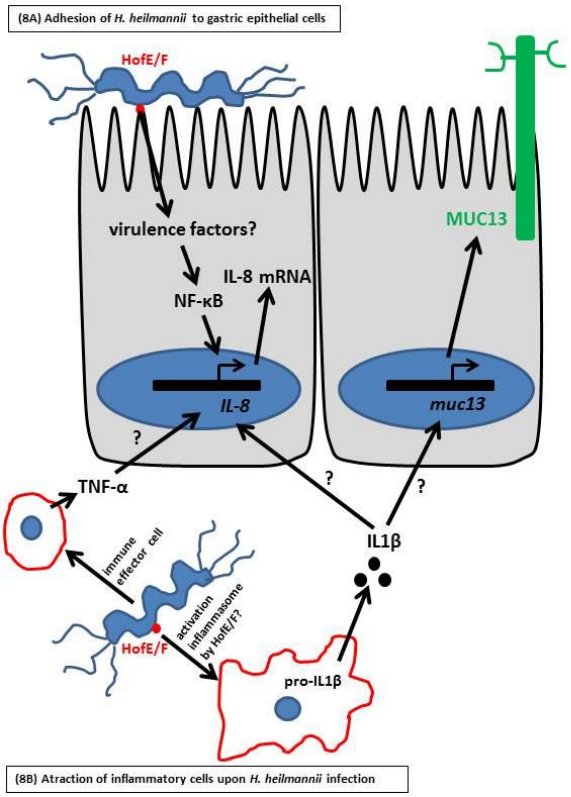


Figure 8: A schematic representation of the hypothetical cascade of events involved in gastric MUC13 expression that occur during adhesion of *H. heilmannii* to epithelial cells.

Shown is the involvement of HofE and HofF OMPs in adhesion of *H. heilmannii* to gastric epithelial cells and the induction of the IL-1β-induced transmembrane MUC13 expression (A). Inflammatory cells responsible for

IL-1β and TNF-α secretion are attracted to the site of *Helicobacter* infection (B). The signaling pathways regulating IL-1β-induced MUC13 and IL-1β- and TNF-α-induced IL-8 expression in the stomach during *Helicobacter* infection remains to be further elucidated.

In gastric epithelial cells, IL-8 expression was also increased by TNF-α. This cytokine can be secreted by immune effector cells which are attracted upon *Helicobacter* infection, as shown before (Sheng et al., 2013; Fusunyan et al., 1998; Figure 8). Additionally, expression of TNF-α was significantly increased in the antrum of the stomach of animals infected with *H. heilmannii* ASB1wt or *H. pylori* SS1. From these results, it can be concluded that expression of IL-8 in the stomach can be regulated by both the IL-1β and TNF-α signaling pathways as has also been shown in intestinal epithelial cells (Sheng et al., 2013; Fusunyan et al., 1998). How TNF-α induces gastric IL-8 expression upon *H. heilmannii* infection remains to be further studied (Figure 8).

The role of *H. heilmannii* HofE, HofF and HofG and *H. pylori* HofF in the stimulation of MUC13 expression was examined *in vitro* and *in vivo*. *H. heilmannii ΔhofE* and *ΔhofF* and *H. pylori ΔhofF* did not induce an increased expression of MUC13 in human gastric epithelial cells and of Muc13 in the stomach of mice. As described above, these mutants also showed a clear and reduced gastric colonization capacity *in vivo*, suggesting that colonization is essential for gastric Muc13 expression. Additionally, these *Helicobacter* mutants did not upregulate expression of IL-1β mRNA neither in the murine stomach. Whether these outer membrane proteins play a role in the activation of the inflammasome for IL-1β expression remains elusive (Figure 8). The above novel findings in *Helicobacter* research highlight the importance of *H. heilmannii* HofE and HofF and *H. pylori* HofF in induction of IL-1β and human MUC13 and murine Muc13 expression in the stomach (Figure 8).

Infection with *H. heilmannii*, *H. pylori* and their mutants all induced a significant increase in IL-8 expression both *in vitro* and *in vivo*. This may suggest that the *Helicobacter* Hof OMPs and human MUC13 and murine Muc13 do not play a major role in IL-8 production in the stomach. This is in contrast to what has been described in the intestinal tract where MUC13 increases IL-8 expression in human intestinal epithelial cells in response to a *Campylobacter* infection (Sheng et al., 2012). Additionally, *H. heilmannii ΔhofE* and *ΔhofF* and *H. pylori ΔhofF* did not induce a significant change in expression of TNF-α highlighting that the increased expression of the human IL-8 related MIP-2, KC and Lix proteins demonstrated in the stomach of mice infected with these mutants is regulated by another signalling pathway. Expression of IL-8 can indeed be regulated by multiple signalling pathways (Hoffmann et al.,

2002).

Taken together, the results of the bacterial *hof* genes and host genes expression experiments, the *Helicobacter* binding assays and the experimental infection studies in Balb/c mice illustrate the importance of the *H. heilmannii hof* locus, in particular genes encoding HofE and HofF, in adhesion to the gastric mucosa as well as the role of *H. heilmannii* HofE and HofF and *H. pylori* HofF in human MUC13 and murine Muc13 expression.

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Table S1: The *hof* gene expression patterns of *H. heilmannii* ASB1wt, ASB1*ΔhofE*, ASB1*ΔhofF* and ASB1*ΔhofG* in response to exposure at pH2, 42°C and H_2O_2 for 30 min and pH7, mucins and cells for 24h. Data are presented as fold change (± standard deviation) in gene expression normalized to two reference genes and relative to the results for *H. heilmannii* grown under normal *in vitro* conditions (pH 5).

pH2_30min						
	hofH	***hofF***	***hofE***	***hofG***	***hofC***	***hofD***
ASB1wt	0.82±0.032	0.84±0.07	0.5±0.09*	0.7±0.015	1.11±0.14	1.11±0.06
ASB1*ΔhofE*	0.58±0.052	2.29±0.41*	-	1.02±0.14	2.52±0.27	2.37±0.44
ASB1*ΔhofF*	0.92±0.19	-	3.67±0.16*	0.85±0.14	0.86±0.18	1.18±0.29
ASB1*ΔhofG*	0.85±0.15	0.56±0.02	1.07±0.17	-	1.02±0.07	1.25±0.22
42°C_30min						
	hofH	***hofF***	***hofE***	***hofG***	***hofC***	***hofD***
ASB1wt	0.38±0.03*	0.59±0.05*	0.39±0.06*	0.24±0.02*	1.06±0.09	0.48±0.07*
ASB1*ΔhofE*	1.04±0.15	1.87±0.08	-	0.94±0.04	0.71±0.11	0.52±0.03
ASB1*ΔhofF*	0.96±0.09	-	1.54±0.43	1.09±0.05	0.71±0.03	0.54±0.09
ASB1*ΔhofG*	0.78±0.07	1.60±0.07	1.37±0.12	-	1.4±0.07	0.77±0.10
H_2O_2_30min						
	hofH	***hofF***	***hofE***	***hofG***	***hofC***	***hofD***
ASB1wt	0.93±0.06	1.23±0.15	0.89±0.01	1.06±0.29	1.25±0.03	0.88±0.02
ASB1*ΔhofE*	0.77±0.09	2.44±0.10*	-	1.03±0.19	1.73±0.21	1.58±0.11
ASB1*ΔhofF*	1.09±0.32	-	5.11±0.48*	0.89±0.02	0.84±0.02	1.19±0.04
ASB1*ΔhofG*	1.22±0.19	0.64±0.05	0.77±0.06	-	1.61±0.12	0.83±0.06
pH7_24h						
	hofH	***hofF***	***hofE***	***hofG***	***hofC***	***hofD***
ASB1wt	2.14±1.46	4.06±2.07*	3.89±1.79*	2.29±0.25*	1.53±0.36	2.17±0.74
ASB1*ΔhofE*	4.86±2.32*	2.88±0.18*	-	2.64±0.23*	1.81±0.08	1.75±0.01
ASB1*ΔhofF*	1.5±0.06	-	3.09±0.07*	2.04±0.29	1.72±0.15	1.79±0.16
ASB1*ΔhofG*	1.34±0.10	2.86±0.4*	2.95±0.12*	-	1.59±0.01	1.16±0.31
mucin co-cultures_24h						
	hofH	***hofF***	***hofE***	***hofG***	***hofC***	***hofD***
ASB1wt	1.9±1.5	2.7±0.18*	3.37±0.15*	3.68±0.13*	4.75±1.48*	2.57±0.12*
ASB1*ΔhofE*	1.54±0.09	0.87±0.09	-	1.25±0.04	1.01±0.04	1.31±0.12
ASB1*ΔhofF*	1.10±0.32	-	0.71±0.19	3.08±0.03*	0.58±0.04	1.58±0.28
MKN7 cells_24h						
	hofH	***hofF***	***hofE***	***hofG***	***hofC***	***hofD***
ASB1wt	1.06±0.35	1.15±0.15	1.26±0.09	1.03±0.31	1.15±0.30	1.41±0.26

ASB1*ΔhofE*	2.15±0.57	0.78±0.26	-	0.74±0.16	1.59±0.5	0.89±0.27
ASB1*ΔhofF*	1.42±0.4	-	0.83±0.03	0.77±0.01	0.71±0.15	0.93±0.05
ASB1*ΔhofG*	1.13±0.12	0.67±0.04	0.61±0.09	-	1.15±0.31	0.5±0.07

* Significant differences in expression level between *H. heilmannii* grown under different environmental conditions and normal *in vitro H. heilmannii* growth are indicated by * $p<0.05$ (ANOVA).

Table S2: Expression of gastric mucins, the human IL-8 related mouse genes (*Mip-2, Kc* and *Lix*), IL-1β and TNFα in the stomach of *H. heilmannii* ASB1wt-, ASB1*ΔhofF*-, ASB1*ΔhofE*-, ASB1*ΔhofG*-, and *H. pylori* P1- and STM138*ΔhofF*-infected and control Balb/c mice. Data are presented as fold change (± standard deviation) in gene expression normalized to 3 reference genes and relative to the results for the negative-control group.

antrum 4 weeks post-infection						
	ASB1wt	**ASB1*ΔhofE***	**ASB1*ΔhofF***	**ASB1*ΔhofG***	**SS1**	**STM138*ΔhofF***
Muc1	1.05±0.1	0.73±0.08	0.87±0.12	1.12±0.13	0.68±0.2	0.87±0.15
Muc5ac	1.02±0.54	0.79±0.19	1.06±0.17	0.49±0.08	1.08±0.46	0.87±0.32
Muc6	0.93±0.04	0.83±0.11	0.92±0.14	1.11±1.04	0.87±0.36	0.93±0.19
Muc13	3.19±0.97*	2.01±0.63	2.15±0.83	3.93±1.72*	1.34±0.78	1.11±0.16
KC	3.87±1.41*	1.29±0.30	1.29±0.49	9.47±2.27*	0.84±0.19	1.05±0.11
Lix	6.63±5.89*	1.04±0.18	1.89±1.81	3.9±0.46*	1.30±0.6	1.4±0.25
MIP-2	2.84±2.55	1.13±0.35	1.10±0.55	2.14±0.69	1.27±1.07	1.31±0.25
TNF-α	4.16±2.68*	0.84±0.15	1.11±0.35	3.96±0.79*	1.65±0.71	1.51±0.72
IL-1β	8.13±5.5*	1.41±0.17	1.49±1.14	7.92±2.09*	2.35±0.7*	2.18±1.02
fundus 4 weeks post-infection						
	ASB1wt	**ASB1*ΔhofE***	**ASB1*ΔhofF***	**ASB1*ΔhofG***	**SS1**	**STM138*ΔhofF***
Muc1	1.11±0.72	1.30±0.66	1.37±0.79	1.35±0.54	1.23±0.40	1.33±0.70
Muc5ac	2.66±2.03	2.37±1.15	1.69±1.27	1.81±1.24	4.5±3.44	4.07±3.39
Muc6	1.34±0.89	1.69±0.51	1.44±0.87	1.46±1.06	2.15±0.37	2.09±1.04
Muc13	1.78±1.14	1.82±0.62	1.75±0.90	0.71±0.13	0.78±0.36	1.19±0.78
KC	2.13±1.04	1.87±0.67	1.62±0.70	1.30±0.29	1.01±0.31	1.73±0.73
Lix	1.39±0.29	2.37±0.68	2.57±0.80	0.87±0.27	1.78±0.69	2.35±0.83
MIP-2	3.18±2.27	4.18±2.24*	4.84±4.03*	5.86±6.34*	11.45±6.93*	8.85±7.07*
TNF-α	0.82±0.62	0.85±0.51	0.87±0.27	0.83±0.22	0.68±0.26	0.65±0.35
IL-1β	1.55±0.45	1.37±0.29	1.32±0.21	1.43±0.1	1.95±0.82	1.49±0.36
antrum 9 weeks post-infection						
	ASB1wt	**ASB1*ΔhofE***	**ASB1*ΔhofF***	**ASB1*ΔhofG***	**SS1**	**STM138*ΔhofF***
Muc1	2.21±0.37	0.57±0.47	1.60±0.28	1.18±0.19	1.06±0.21	0.83±0.15
Muc5ac	0.85±0.41	2.94±2.46	1.01±0.29	0.48±0.24*	0.49±0.19*	0.55±0.20*
Muc6	1.32±0.33	1.06±0.38	2.86±3.57*	0.49±0.15*	1.94±2.37*	0.60±0.14*
Muc13	6.66±2.29*	0.75±1.05	2.22±1.70	3.78±1.33*	2.55±0.96*	1.03±0.31
KC	12.5±3.94*	0.57±0.79	3.7±3.31*	5.86±2.3*	2.77±3.96*	0.78±0.31
Lix	5.23±2.13*	0.40±0.6	2.5±2.83	4.07±1.63*	0.73±0.33	0.39±0.23
MIP-2	1.29±0.62	0.56±0.22	0.72±0.42	0.47±0.28	0.46±0.19	0.46±0.14

TNF-α	9.56±2.51*	1.95±0.84	2.17±0.54	8.37±3.09*	5.2±4.08*	1.65±1.4
IL-1β	5.1±2.97*	0.86±0.51	1.19±0.59	5.04±0.55*	1.73±0.95	1.18±0.61

fundus 9 weeks post-infection

	ASB1wt	**ASB1*ΔhofE***	**ASB1*ΔhofF***	**ASB1*ΔhofG***	**SS1**	**STM138*ΔhofF***
Muc1	1.29±0.38	1.29±0.86	2.44±1.20	2.06±1.02	2.69±1.61	2.22±0.68
Muc5ac	1.44±0.94	1.80±1.32	1.60±1.01	2.17±1.97	2.24±1.96	1.58±1.02
Muc6	2.12±0.31*	3.12±1.57*	2.62±0.58*	2.42±1.46*	2.81±1.56*	2.80±1.66*
Muc13	2.14±0.25*	1.3±0.25	2.02±0.79	2.12±1.16*	3.03±0.89*	2.02±0.80
KC	2.27±0.35*	2.73±1.27*	4.76±3.30*	2.79±1.62*	2.81±1.02*	3.21±1.27*
Lix	1.19±0.85	4.98±2.05*	4.10±2.36*	4.25±2.34*	4.77±2.21*	7.19±3.02*
MIP-2	1.61±1.89	1.70±1.77	1.25±2.00	2.62±3.43	6.04±4.16*	6.30±4.11*
TNF-α	1.31±0.46	0.85±0.42	0.87±0.46	1.31±0.64	1.15±0.66	1.14±0.3
IL-1β	0.96±0.39	0.55±0.13	0.53±0.25	0.96±0.35	1.01±0.3	0.77±0.53

***** Significant differences in expression level between the infected groups and the negative-control group at a certain time-point are indicated by * $p<0.05$ (ANOVA).

Table S3: List of primers used in quantitative RT-PCR for gene expression.

Primer*	Sequence	Reference
H2afz-F (m)	5’- GGT ATC ACC CCT CGT CAC TT-3’	1
H2afz-R (m)	5’- TCA GCG ATT TGT GGA TGT GT-3’	1
HPRT-F (m)	5’- CAG GCC AGA CTT TGT TGG AT-3’	1
HPRT-R (m)	5’- TTG CGC TCA TCT TAG GCT TT-3’	1
PPIA-F (m)	5’- AGC ATA CAG GTC CTG GCA TC-3’	1
PPIA-R (m)	5’- TTC ACC TTC CCA AAG ACC AC-3’	1
Muc1-F (m)	5’- GGT TGC TTT GGC TAT CGT CTA TTT-3’	1
Muc1-R (m)	5’- AAA GAT GTC CAG CTG CCC ATA-3’	1
Muc6-F (m)	5’- TGC TCC CAG AAT GAG TAC TTC GA-3’	1
Muc6-R (m)	5’- CAG AGG TGG AAC TGT GAA ACT CAG T-3’	1
Muc13-F (m)	5’- GCC AGT CCT CCC ACC ACG GTA-3’	1
Muc13-R (m)	5’- CTG GGA CCT GTG CTT CCA CCG-3’	1
KC-F (m)	5’- GCT GGG ATT CAC CTC AAG AA -3’	2
KC-R (m)	5’- TCT CCG TTA CTT GGG GAC AC -3’	2
MIP2-F (m)	5’- TGC CTG AAG ACC CTG CCA AGG-3’	2
MIP2-R (m)	5’- GTT AGC CTT GCC TTT GTT CAG-3’	2
Lix-F (m)	5’- CTC AGT CAT AGC CGC AAC CGA GC-3’	2
Lix-R (m)	5’- CCG TTC TTT CCA CTG CGA GTG C-3’	2
HH_FWQ (Hh)	5’- CTT TCT CCT GGT GAA GTG ATT CTC -3’	1
HH_RVQ (Hh)	5’- GCT GTA CCA GAG GCA ATG TCC AAG-3’	1
Hpyl_F1 (Hp)	5’- AAA GAG CGT GGT TTT CAT GGC G-3’	1
Hpyl_R1 (Hp)	5’- GGG TTT TAC CGC CAC CGA ATT TAA-3’	1
GADPH_F (h)	5’- CCT GTA CGC CAA CAC AGT GC-3’	3
GADPH_R (h)	5’- ATA CTC CTG CTT GCT GAT CC-3’	3
MUC13_F (h)	5’- AGC CCT TTC TGC GTT GCT TCC A-3’	3
MUC13_R (h)	5’- CAC TCA GGG GCC CCA CCA CT-3’	3
IL8_F (h)	5’- AAG AGA GCT CTG TCT GGA CC-3’	3
IL8_R (h)	5’- GAT ATT CTC TTG GCC CTT GG-3’	3
atpA-HH-F (Hh)	5’- CCT TGA AGA GGG CTG TAT CG-3’	This study
atpA-HH-R (Hh)	5’- ACG GGG ACT TTC ATG AGT TG-3’	This study
efp-HH-F (Hh)	5’- GTT CAT GGA CAC GGA AAC CT-3’	This study

efp-HH-R (Hh)	5’- ACT TGA GAG CCA TCG AGC AT-3’	This study
hofH-HH-F (Hh)	5’- ACC TAC ATT TGG AGC GAT GC-3’	This study
hofH-HH-R (Hh)	5’- CCT TGG GTT TTC CCA CTT CT-3’	This study
hofC-HH-F (Hh)	5’- ACG ACA CCA CAC AGG TCA AA-3’	This study
hofC-HH-R (Hh)	5’- GGA GCT GAA CAC TTG GAA GG-3’	This study
hofD-HH-F (Hh)	5’- ATC TGC GTC TAG CGA CTG GT-3’	This study
hofD-HH-R (Hh)	5’- TAG GGG TTG ACC TTC CAG TG-3’	This study
hofE-HH-F (Hh)	5’- GCT ACA CCG AAG GCT TTG AG-3’	This study
hofE-HH-R (Hh)	5’- GCC CTG CTT GTT CTT AGT CG-3’	This study
hofF-HH-F (Hh)	5’- GCA GAT TGT GTG CAA GGC TA-3’	This study
hofF-HH-R (Hh)	5’- CAT AAC GTC CGC CTC TGA AT-3’	This study
hofG-HH-F (Hh)	5’- AAT TAT CCG GGG CTT ACC AC-3’	This study
hofG-HH-R (Hh)	5’- GGA CCC GCT CAT AAA ATC AA-3’	This study
efp-HP-F (Hp)	5’- GAT GAG CGA GCT CAA AAA GG -3’	This study
efp-HP-R (Hp)	5’- GGG CTT GAC ATG CTG GTA TT -3’	This study
Urel-HP-F (Hp)	5’- CCA GCG ACT GGG TTA TTG TT -3’	This study
Urel-HP-R (Hp)	5’- GGA TAA AAT CGC AGC AGG AA -3’	This study
hofH-HP-F (Hp)	5’- CAT TAG GGT TGC GCC TTT TA -3’	This study
hofH-HP-R (Hp)	5’- CGC CAA AGA GAT TCC CAA TA -3’	This study
hofC-HP-F (Hp)	5’- ACA CTT GGG ATC CGT TCT TG -3’	This study
hofC-HP-R (Hp)	5’- TAA GCA CCA CCA ACC ACA AA -3’	This study
hofD-HP-F (Hp)	5’- AAC CTC GTT TAG CCA TGG TG -3’	This study
hofD-HP-R (Hp)	5’- AAG CCT TCA ACC CCT TGA GT -3’	This study
hofE-HP-F (Hp)	5’- TCC ACC AAC GCT TTG ACT -3’	This study
hofE-HP-R (Hp)	5’- ACC ATA CCA GCC AAT CCT TG -3’	This study
hofF-HP-F (Hp)	5’- AGG GCT TTA CCC TAC CGA AA -3’	This study
hofF-HP-R (Hp)	5’- AAG GCT CCC GTC TAA ATG GT -3’	This study
hofG-HP-F (Hp)	5’- CGA TTG CAG CCA TTC CTT AT -3’	This study
hofG-HP-R (Hp)	5’- AGC CCC CAA TGA AAT TCT CT -3’	This study

m: mouse; h: human; Hh: *H. heilmannii*; Hp: *H. pylori*; *Primers used for measuring mRNA expression of Muc5AC were purchased from QIAGEN Cat.no.:Qt01196006

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GENERAL DISCUSSION

..."What we know is a drop, what we don't know is an ocean!"... by Isaac Newton

Gastric NHPH species from domestic animals have been associated with disease in humans. Some distinct features, such as the association with gastric MALT lymphoma, indicate that these zoonotic bacteria should not be considered as a "light" version of *H. pylori*. Information on how NHPH species colonize the stomach and cause disease is still scarce. The recent successful *in vitro* isolation of several of these species, including *H. heilmannii*, has opened new perspectives for understanding the pathogenesis of NHPH-associated gastric pathology in humans.

Effect of a H. heilmannii *infection on the expression of gastric mucin-types*

Several gastric *Helicobacter* species are able to colonize the mucus niche of the human gastric mucosa. The main components of the mucus layer are heavily glycosylated mucins which interact with *Helicobacter* (Kaneko et al., 2003; Chen et al., 2004; Shirazi et al., 2000). Three mucin types are highly expressed in a healthy gastric mucosa. The membrane-bound MUC1 mucin is detected in mucous cells of the surface epithelium and in the pyloric and fundic glands of the stomach (Ho et al., 1995; Peterson, 1991; Reis et al., 1998). Secreted MUC5AC is found in the foveolar epithelium of both antrum and fundus (Ho et al., 1995; Audie et al., 1993) whereas secretion of MUC6 is limited to mucous neck cells of the fundic and pyloric glands (Ho et al., 1995; Bartman et al., 1998; De Bolos et al., 1995). One of the hallmarks of human gastric pathologies are the alterations in mucin distribution and expression in the stomach (Skoog et al., 2012; Weis et al., 2013). It has been reported that a *H. pylori* infection changes gastric mucin expression in humans (Ho et al., 1995; Wang and Fang, 2003; Babu et al., 2006; Beil et al., 2000; Byrd et al., 2000; Morgenstern et al., 2001; Fichman et al., 2004; Kim et al., 2003). This has also been confirmed by experimental *H. pylori* infections in Rhesus monkeys and rodents (Linden et al., 2008; Navabi et al., 2013). Both animal models have been shown to be suitable to study *Helicobacter*-related gastric pathology in humans (O'Rourke and Lee, 2003). On the contrary, when we started our studies, no information was available about mucin expression and distribution in the stomach of NHPH infected patients and only few studies dealt with the gastric mucin dynamics in animals experimentally infected

with animal-associated gastric *Helicobacter* species. Furthermore, these were limited to *H. felis* (Nomura et al., 2004; Kurt-Jones et al., 2007; Schmitz et al., 2009).

In the first chapter of this thesis, we describe the impact of a *H. heilmannii* infection on the distribution and expression of mucins in the stomach of mice. In this study, *H. pylori* was included for comparison. In the first 9 weeks post-infection, expression of the gland mucin Muc6 was significantly increased in both the antrum and the fundus of the stomach of *H. heilamnnii*- and *H. pylori*-infected mice. Expression of gastric Muc1 and Muc5AC was unaffected. During this acute stage of infection, these animals developed gastritis particular in the fundus of the stomach. Similar findings have been reported in *H. pylori*-associated gastritis in humans (Beil et al., 2000; Byrd et al., 2000; Matsuzwa et al., 2003; Van den Brink et al., 2000). Although it has been shown that *H. pylori* interacts with MUC1 and MUC5AC by binding to sLe^x and Le^b, respectively, expression of MUC5AC and MUC1 seems not to be affected by a *H. pylori* infection (Marques et al., 2005; Kang et al., 2008). Unpublished results of recent *in vitro* mucin binding assays showed that *H. heilmannii* does not adhere to sLe^x and Le^b. Mucins can carry on the order of 100 different carbohydrate structures (Klein et al., 1993). Whether MUC1 and/or MUC5AC carry other glycans, that could interact with *H. heilmannii*, remains to be further studied.

Gastric glands of *H. pylori*-infected patients have a low degree of colonization with this bacterium (Kawakubo et al., 2004), although increased expression of MUC6 was seen in these patients, as was also shown in our *in vivo* experiment. This finding may be attributed to the involvement of α1,4-linked N-acetylglucosamine, present on MUC6, in the clearance of *H. pylori* from the glands. This carbohydrate structure has a strong antibacterial activity against *H. pylori* by inhibiting its biosynthesis of cholestryl-α-D-glucopyranoside (Kawakubo et al., 2004). It has therefore been suggested that MUC6 plays a role in the host defense against *H. pylori* infection (Kawakubo et al., 2004). Reduced MUC6 expression will increase gastric mucosal susceptibility to *H. pylori* infection. Indeed, a decrease in expression or even loss of MUC6 has been described in more severe gastric pathologies, including intestinal metaplasia and adenocarcinoma (Byrd et al., 2000; Zheng et al., 2006).

On the contrary, in our study, the increased Muc6 expression in the first 9 weeks post-infection was positively correlated with the increased number of *H. heilmannii* bacteria. Unlike *H. pylori* which is mainly found at the surface epithelium, *H. heilmannii* can additionally be seen more deeply in the glands of the gastric mucosa (unpublished results). From 9 weeks post-infection onwards, expression of Muc6 was found to adopt the normal

expression pattern as in the uninfected animals. These findings suggest a potential role of Muc6 in the initial colonization of the gastric mucosa by *H. heilmannii* rather than a role in host defense as shown for *H. pylori*.

Effect of a* H. heilmannii *infection on the expression of non-gastric mucin-types

During our *in vivo* experiment, significantly increased expression of non-gastric mucin-types, including Muc5B, Muc4 and Muc13, was found in the murine stomach in response to *H. heilmannii* and *H. pylori* infection.

In humans, MUC5B is predominantly found in both saliva and the respiratory tract. MUC5B is not expressed in the healthy human stomach but its expression has been shown to be upregulated in human gastric adenocarcinomas (Schmitz et al., 2009). In our study, the mRNA expression of murine Muc5B remained high in the stomach as gastric disease progressed in severity during later stages of *heilmannii* and *H. pylori* infection. This resembles what has been seen in human gastric disease pointing out the role of MUC5B in disease progression. Expression of this mucin in the human stomach is regulated by IL-1β and IL-17A in an NF-κB dependant manner (Fujisawa et al., 2011). Furthermore, MUC5B is able to bind SabA expressing *H. pylori* strains at neutral pH (Linden et al., 2008). The upregulation of MUC5B seen in the human stomach may thus also allow for increased binding of *Helicobacter*. *H. heilmannii*, however, lacks the SabA adhesin. Whether this bacterium is also able to bind MUC5B via other adhesins remains to be further determined.

Besides MUC5B, also MUC4, a transmembrane mucin which is normally expressed in the intestines, plays a crucial role in the progression to gastric malignancies, such as mucous metaplasia (Schmitz et al., 2009). A number of studies, including our own *H. heilmannii* experimental infection study in mice, show that the loss of parietal cells leads to the evolution of SPEM, a gastric fundic metaplastic lineage consisting of TFF2-expressing mucous cells with Brunner's gland or deep antral gland morphology (Nomura et al., 2004; Wang et al., 1998; Goldenring et al., 2000). Gastric MUC4 expression has been shown to be a marker for SPEM (Weis et al., 2013). The molecular mechanisms for its involvement in this type of metaplastic progression may be related to the epidermal growth factor (EGF)-like domain of MUC4 that is known to interact with the receptor tyrosine kinase ErbB2 and alter epithelial cell growth (Schmitz et al., 2009; Jonckheere et al., 2004). Furthermore, MUC4 has been shown to be upregulated by IL-6, via the gp130/STAT3 pathway and the transforming growth factor b (TGFb), in *H. pylori*-infected gastric epithelial cells, highlighting the role of these

signal transducers in gastric tumorigenesis (Mejias-Luque et al., 2008). Increasing evidence in humans and rodent models suggests that intestinal metaplasia, another mucous metaplastic lineage and associated with intestinal MUC2 expression in the stomach, develops in the presence of pre-existing SPEM. This supports the concept that SPEM is a neoplastic precursor in the carcinogenesis cascade (Yoshizawa et al., 2007; Nam et al., 2009; Goldenring et al., 2010). In our study, intestinal metaplasia was not observed. Further studies are necessary in which *H. heilmannii*-infected mice are followed during more than a year to determine if SPEM will further differentiate into intestinal metaplasia and eventually dysplasia, an early stage in the development of cancer. MUC13, another transmembrane mucin, is normally expressed on the apical surface of all cells in glands and villi of the small intestine. It is also expressed by enterocytes and goblet cells throughout the large intestine where both cell-surface (highest in the surface epithelium) and cytoplasmic localization has been described (Sheng et al., 2011). Additionally, this cell-surface mucin is abundantly present in the trachea and kidneys (Williams et al., 2001) whereas in the normal stomach, its expression level is very low (Williams et al., 2001; Maher et al., 2011; Sheng et al., 2011). MUC13 contains a N-terminal signal peptide followed by a large serine-threonine rich tandem repeat domain. The central region of MUC13 includes three EGF-like domains (EGF1, EGF2 and EGF3), suggesting that MUC13 plays an important role in the signalling cascade. Additionally, a sea urchin sperm protein enterokinase arginine (SEA) module is present between EGF1 and EGF2, providing a cleavage site which separates MUC13 into an extracellular α subunit and a transmembrane β subunit (Williams et al., 2001; Shimamura et al., 2005). In the intestines, MUC13 plays an essential role in limiting tissue injury after exposure to environmental toxins through inhibition of apoptosis and the maintenance of intestinal epithelial barrier function (Sheng et al., 2011). It has been suggested that the protective role of MUC13 in inflammation is most likely due to cytoplasmic domain signalling (Moehle et al., 2006; Sheng et al., 2011). On the contrary, polymorphisms that impair MUC13 expression and signalling could lower this level of protection (Moehle et al., 2006). It should therefore be noted that sustained elevation of MUC13 may promote the transition from chronic inflammation to cancer (Kufe, 2009). Indeed, aberrant expression of MUC13 has been described in ovarian, pancreatic, gastric and colorectal cancers (Maher et al., 2011; Walsh et al., 2007; Shimamura e al., 2005; Chauhan et al., 2007). In pancreatic cancer, increased MUC13 expression has been correlated with tumorigenic characteristics including repression of p53 and the upregulation of ErbB2, p21-activated kinase 1 (PAK1), extracellular signal-regulated kinase (ERK), Akt and

metastasin. Conversely, suppression of MUC13 resulted in the suppression of these tumorigenic characteristics (Chauhan et al., 2007). Based on these findings, MUC13 may be involved in cancer pathobiology and could be a potential diagnostic/prognostic biomarker of cancer as well as a target for antibody guided therapy for cancer treatment (Byrd et al., 2004; Packer et al., 2004).

In our *in vivo* experiment, aberrant murine Muc13 expression was noticed already in the early stage of infection in the stomach of *H. heilmannii* and *H. pylori*-infected mice and its expression remained high when chronic inflammation evolved into MALT-lymphoma-like lesions. This latter finding provides further evidence for the role of MUC13 in cancerogenesis as described above. However, the increased expression of Muc13 in the first 9 weeks post-infection was positively correlated with the increased number of *H. heilmannii* and *H. pylori* bacteria in the stomach. This finding, which is novel in *Helicobacter* research in general, suggests an additional role for Muc13 in the initial colonization of the gastric mucosa by *Helicobacter*.

Pathways involved in gastric MUC13 expression

The pathway regulating gastric MUC13 expression was further investigated in the second chapter of this thesis.

In the intestines, MUC13 expression is regulated by IL-22 through the activation of the signal transducers STAT3 and cytokine receptor-associated JAK1, which play crucial roles in epithelial proliferation, differentiation, and apoptosis (Begue et al., 2011; Heneghan et al., 2013; Okamoto et al., 2011). IL-22, expressed by innate and adaptive immune system cells, is a key cytokine for gut epithelial homeostasis and mucosal repair, underlining the protective role of MUC13 in inflammation in the intestines (Fernandes et al., 2014).

In our study, however, we found that gastric MUC13 expression is induced by the pro-inflammatory cytokine IL-1β. This cytokine enables *H. heilmannii* and *H. pylori* to colonize the gastric mucosa by inhibiting acid secretion (Joosten et al., 2013; El-Omar, 2001; Semper et al., 2014). Secretion of IL-1β induced by *H. pylori* depends on the activation of the inflammasome, an intracellular multiprotein oligomer implicated in the activation of inflammatory processes (Semper et al., 2014; Koch et al., 2015). Inflammasome activation in dendritic cells by *H. pylori* infection requires the nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3 (NLRP3) as well as induction of toll-like receptor 2 (TLR2) signalling (Koch et al., 2015). Furthermore, it has been shown that pro-IL-

1β expression is induced by *H. pylori* LPS, while the urease B subunit of *H. pylori* is required for NLRP3 inflammasome licensing (Koch et al., 2015). Additionally, another study highlighted the involvement of the *H. pylori* cagPAI and VacA virulence factors in inflammasome activation (Semper et al., 2014). Both these virulence factors are, however, absent in *H. heilmannii* and it is currently unknown how this bacterium triggers secretion of IL-1β. Screening of a *H. heilmannii* transposon mutant library for its ability to activate the inflammasome may help to identify the virulence factors involved in this process.

It has previously been shown that the intestinal mucin-type MUC2 is activated by IL-1β through the NF-κB signalling pathway in gastric epithelial cells (Mejias-Luque et al., 2010). Whether NF-κB or other signal transducers are involved in the activation of gastric IL-1β-induced MUC13 expression remains to be further elucidated.

Based on the findings described above, it is clear that MUC13 expression in the gastrointestinal tract can be regulated by different signalling pathways.

Another remarkable difference between gastric and intestinal epithelial cells is the involvement of MUC13 in IL-8 signalling in response to inflammation. MUC13 increases IL-8 production in the supernatant of human intestinal epithelial cells in response to a *Campylobacter* infection (Sheng et al., 2013). In the stomach, IL-8 is also secreted in the supernatant of epithelial cells infected with *H. pylori* (Lee et al., 2013) and *H. heilmannii* (our study). However, we demonstrated that the expression of this cytokine is not regulated by MUC13. Gastric IL-8 expression seems to be induced by the transmembrane mucin MUC1, as shown before (Sheng et al., 2013).

Adhesion of *H. heilmannii* to the gastric mucosa

A critical step in gastric *Helicobacter* colonization is adhesion to the gastric mucosa. For *H. pylori*, it has been described that the majority of the bacteria resides in the mucus layer by binding to mucins while only a small percentage adheres to the gastric epithelium underneath the mucus layer (McGuckin et al., 2011). *H. heilmannii* is also able to bind to human gastric mucins and epithelial cells, although to a lesser extent than *H. pylori* (Joosten et al., 2015). The fact that man is not the natural host for *H. heilmannii* might explain the lower binding capacity to the human gastric mucosa. Indeed, *H. heilmannii* adhered to a higher extend to the feline than to the murine mucosal surface epithelium. The latter resembles the human gastric mucosa (Joosten et al., 2015). The ability of *H. pylori* and *H. heilmannii* to bind to gastric mucins and epithelial cells is pH dependent. Binding to gastric mucins and epithelial cells was

the highest at pH2 and pH7, respectively (Joosten et al., 2015). This is consistent with the physiological pH gradient in the stomach ranging from pH 1-2 in the gastric lumen (where most mucins reside) to pH 6-7 at the epithelial cell surface (Linden et al., 2008; Skoog et al., 2011). In addition, unpublished results of recent *in vitro* assays showed that the binding pattern of *H. heilmannii* to human gastric mucins is distinctly different from that of *H. pylori*. Binding of *H. heilmannii* was higher to mucins from healthy individuals and in particular to a mucin sample containing high amounts of N-terminal acetylgalactosamine β1-4N-acetylglucosamine (LacdiNAc). This structure is involved in the termination of glycan side chains, which has been suggested to prevent more complex glycosylation (Kenny et al., 2012). The availability of less complex glycan structures may be beneficial for *H. heilmannii* adhesion, as there would then be less steric hindrance to interfere with binding to other possible ligands. Alternatively, LacdiNac might be a ligand itself that *H. heilmannii* can bind to. Binding assays using ELISA with this carbohydrate structure immobilized on polystyrene 96-well microtiter plates may clarify this matter.

In the human stomach, the expression of LacdiNac is absent in cardiac glands. It is expressed at the surface of the fundic mucosa and in the distal part of the pyloric glands. This carbohydrate structure seems to be co-localized with MUC5AC, but has also been found deeper into the glands, suggesting that other mucin types may express LacdiNac as well (Rossez et al., 2014). It remains thus to be further elucidated, for instance by double immunostaining, which other mucin-types carry LacdiNac .

Bacterial OMPs are directly involved in the interaction of pathogenic bacteria with their host (Galdiero et al., 2012). *H. pylori* is equipped with a large set of OMPs, which role in the adhesion process has been well-studied. The *H. pylori* OMPs BabA/B (HopS/T), SabA (HopP), AlpA/B (HopB/C), OipA (HopH), HopZ, HopQ and HomB have been identified as adhesins (Oleastro and Menard, 2013). The Bab and Sab adhesins are involved in adhesion to gastric mucins (Oleastro and Menard, 2013; Ilver et al., 1998; Mahdavi et al., 2002) whereas the other identified *H. pylori* adhesins have been described to play a role in binding to gastric mucus-secreting epithelial cells (Oleastro and Menard, 2013). *In silico* analyses of the *H. heilmannii* genome (Smet et al., 2013) revealed the presence of 53 OMPs which is in agreement with the number of OMPs found in *H. pylori*. Comparative genomics between the *H. heilmannii* and *H. pylori* genomes showed that only few of these *H. heilmannii* OMPs can be classified among the 5 OMP families (Hop, Hor, Hom, Hof, iron-regulated OMPs and efflux pump OMPs) described in *H. pylori* (Joosten et al., 2015). Only homologs of the *H.*

pylori Hof OMPs seem to be well conserved in *H. heilmannii* but also among other gastric NHPH. (Joosten et al., 2015). *H. heilmannii* lacks all *H. pylori* adhesins described so far (Joosten et al., 2015) indicating that both species harbour a different repertoire of adhesins. In the second chapter of this thesis, we identified the *H. heilmannii* HofE and HofF OMPs as adhesins involved in binding to the gastric mucosa albeit with a higher affinity for epithelial cells than mucins.

Mucins can carry in the order of 100 different carbohydrate structures. The absence of carbohydrates having a strong binding affinity for *H. heilmannii* might explain the low binding capacity of this pathogen to the mucin samples used in our study. The use of carbohydrate-based micro-array platforms may allow to identify interactions of *H. heilmannii* HofE and HofF with specific, known carbohydrate structures.

Epithelial cells can carry extracellular matrix (ECM) molecules (e.g. laminin, type IV collagen, fibronectin and vitronectin) which also can act as receptors for bacterial pathogens (Senkovich et al., 2011). *H. pylori* is able to bind to several host ECM molecules, in particular laminin, which is present on gastric epithelial cells. Binding to laminin is mediated by the AlpA/AlpB Hop adhesins of *H. pylori* (Senkovich et al., 2011). Whether *H. heilmannii* is able to adhere to ECM molecules and whether its HofE/F adhesins are involved remains thus to be further elucidated.

To further characterize the complete adhesin repertoire of *H. heilmannii*, the OMP expression pattern of this microorganism in response to adherence to the gastric mucosa at different pH environments should be studied as well. This can be enabled through whole transcriptome analyses, chromosome insertional mutagenesis approaches and *in vitro* binding assays. OMPs that are altered in expression in relation to adherence can potentially play a role in the adhesion process as has been described before (Skoog et al., 2012).

It has previously been shown that *H. pylori* OMPs, such as the OipA, AlpA/B, HomB, BabA and SabA adhesins albeit via mucin binding or by direct adherence to gastric epithelial cells, are involved in the induction of expression of IL-8 in the gastric epithelium (Oleastro & Menard, 2013; Sheng et al., 2013). In chapter 2, however, it was found that the *H. heilmannii* HofE and HofF adhesins do not play a major role in IL-8 production, but are essential for the activation of IL-1β which on its turn induces expression of MUC13 in the gastric epithelium. Also the *H. pylori* HofF protein was shown to be involved in IL-1β-induced MUC13 expression. Whether this OMP plays a role in *H. pylori* adherence to the gastric mucosa is currently unknown.

Further unravelling the involvement of these *Helicobacter* Hof outer membrane proteins in IL-1β-induced gastric MUC13 signalling is essential for understanding the exact role of this mucin in the host innate immune response during initial *Helicobacter* colonization.

Final conclusions and future perspectives

In conclusion, the results described in this thesis clearly demonstrate that both an acute and chronic *H. heilmannii* infection in mice induce changes in the distribution and expression of mucins in the stomach, in particular Muc6 and Muc13 which were shown to be involved in *H. heilmannii* colonization. Whether *H. heilmannii* is able to bind to carbohydrate structures present on MUC6 and MUC13 remains to be further elucidated. This can, for instance, be done by performing first *in vitro* adhesion studies with immobilized MUC6 and MUC13. If *H. heilmannii* indeed binds to MUC6 and/or MUC13, the carbohydrates present on these mucins can then be released by reductive β-elimination and analysed by liquid chromatography-mass spectrometry. Finally, *in vitro* binding assays with the characterized carbohydrates may allow to identify the receptors for the *H. heilmannii* adhesins.

One of the very first steps in the pathogenesis of gastric *Helicobacter* infections is adhesion of these bacteria to the gastric mucosa. Here, we identified two novel *H. heilmannii* adhesins, HofE and HofF, which also seem to be involved in MUC13 signalling.

Modulating adhesin gene expression is essential for *H. heilmannii* to adapt its adherence properties accordingly to maintain infection in its host. This can be facilitated by bacterial genome plasticity, as has been shown for *H. pylori* (Kang and Blaser, 2006). Genome plasticity, caused by high mutation and recombination events, results in high sequence diversity and population heterogeneity among bacterial strains (Moreli et al., 2010). *H. pylori* and *H. heilmannii* have numerous simple sequence repeats in their genomes which can contribute to genome plasticity (Janulczyk et al., 2010; unpublished results). In *H. pylori*, expression of genes encoding adhesins can be altered through phase variation via slipped-strand mispairing of these simple sequence repeats or via gene conversion (Aberg et al., 2014). How *H. heilmannii* employs genetic variation to regulate adhesin gene expression has not yet been investigated. Therefore, future studies are needed to unravel the genetic mechanisms regulating *hofE* and *hofF* gene expression and to investigate the impact of these mechanisms on gastric MUC13 expression. Such studies may include a screening of available *H. heilmannii* genomes to identify the presence of simple sequence repeats in *hofE* and *hofF* which may result in slipped-strand mispairing. It should also be elucidated whether *hofE* and

hofF genes share a high nucleotide identity at their 5' and 3' end which could allow homologous recombination between these genes. This latter phenomenon is called gene conversion. If simple sequence repeats are found in the coding sequence or promoter region, amplified fragment polymorphisms analyses can be performed to investigate whether *hofE* and *hofF* gene expression is controlled by phase variation through slipped strand mispairing of these simple sequence repeats. An intragenic repeat length in frame with the coding sequence will result in protein expression (ON status) while a repeat length out of frame causes a frameshift and will switch off the protein (OFF status). Intergenic simple sequence repeats might influence transcription by changing the spacing of promoter elements. In case *hofE* and *hofF* share a high nucleotide homology at their 5' and 3' end, fluctuation assays can then be carried out to investigate whether expression is determined by gene conversion.

Subsequently, *H. heilmannii* strains showing differences in length of the identified simple sequence repeats in *hofE* and *hofF* or in which gene conversion has occurred may be further screened for adhesin expression at RNA and protein level. The ones displaying distinct HofE and HofF expression can then be analyzed for their ability to induce MUC13 expression in gastric epithelial cells.

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SUMMARY

Helicobacter pylori is by far the most explored *Helicobacter* species in humans. Infection with this agent has been associated with a variety of gastric pathologies such as gastritis, ulcers and cancer.

Besides *H. pylori*, gastric disease in humans has been associated with other spiral-shaped Non-*Helicobacter pylori Helicobacter* species (NHPH) naturally colonizing the stomach of animals such as *H. heilmannii*. This zoonotic agent, highly prevalent in cats and dogs, has been associated with gastritis, gastric and duodenal ulcers and Mucosa-associated lymphoid tissue (MALT) lymphoma in humans. The risk of developing MALT lymphoma is higher after infection with NHPH than with *H. pylori*. *H. heilmannii* has been described in 8-19% of gastric biopsy samples of humans with severe gastric complaints and with histological evidence of a NHPH infection. However, this is most probably an underestimation of its true prevalence since diagnosis of infections with this agents is difficult and it can also not be excluded that *H. heilmannii* infections sometimes remain unapparent or cause only mild disease signs which are often not thoroughly examined. The prevalence of *H. pylori* in humans from the western world is decreasing, leaving a niche for possible infection with NHPH. Living in close contact with cats and dogs has been identified as a significant risk factor for these infections in humans.

Initially, information on the pathogenesis of human gastric disease associated with an *H. heilmannii* infection was scarce. This was mainly due to its fastidious nature, hampering isolation and cultivation under laboratory conditions. The first successful *in vitro* isolation of *H. heilmannii* from feline stomachs was done in 2011, opening new doors to investigate the role of this pathogen in human gastric pathology.

The human gastric mucosa is covered by a layer of continuously secreted mucus that washes away trapped particles. It is one of the first barriers *Helicobacter* encounters and consists of heavily glycosylated secreted mucins. The gastric epithelium underneath the mucus layer consists of various cell types.

For *H. pylori*, it is known that infection with this pathogen causes alterations in the expression pattern and distribution of gastric mucins as well as a disruption of the gastric mucosa by inducing epithelial cell loss. Loss of parietal cells can lead to mucous metaplasia which is characterized by the presence of non-gastric mucin types.

Similar to *H. pylori*, *H. heilmannii* is able to bind to human gastric mucins as well as to gastric epithelial cells. Both species are mainly observed close to the surface epithelium, but *H. heilmannii* can additionally be seen in close association with acid-secreting parietal cells located in the gastric glands.

At the onset of this thesis, no information was available about the mucin dynamics in the stomach during infection with *H. heilmannii* and how this bacterium is able to adhere to the human gastric mucosa which is essential to initiate and maintain infection.

In our first study, we investigated the expression pattern and distribution of mucins in the stomach of mice at several time points during a one-year infection with *H. heilmannii*, by which gastric disease progressed from gastritis to MALT-lymphoma-like lesions. Markers for acid production by parietal cells and mucous metaplasia were also examined. A mouse-adapted *H. pylori* strain was included for comparison which has been shown to induce MALT lymphoma-like lesions in mice.

In the first 9 weeks post-infection, the mRNA expression of Muc6 was clearly upregulated in both the antrum and fundus of the stomach of *H. heilmannii*- and *H. pylori*-infected mice. Interestingly and novel in *Helicobacter* research, Muc13 was upregulated already at 1 day post-infection in the fundus of the stomach of *H. heilmannii* and *H. pylori*-infected mice. Its expression level remained high in the stomach over the course of the infection. Muc13 is, however, not expressed in a healthy stomach, and a high expression of this mucin was so far only described in gastric cancer. Additionally, the increased expression of both Muc6 and Muc13 at this acute stage of infection was positively correlated with the increased number of *H. heilmannii* bacteria in the stomach, highlighting a role for these mucins in the initial colonization of the gastric mucosa by *H. heilmannii*. In *H. pylori*-infected mice, an increased number of *H. pylori* bacteria was positively correlated with an increased Muc13 expression but not with an increased Muc6 expression.

In the later stages of *H. heilmannii* infection, mRNA expression of H^+/K^+ ATPase α/β and KCNQ1 (markers for acid production) decreased, whereas the expression of Muc4, Tff2, Dmbt1 and PigR (markers for mucous metaplasia) increased starting at 16 weeks post-infection, suggesting the existence of spasmolytic polypeptide-expressing metaplasia in the fundus of the stomach of *H. heilmannii*-infected mice. Mucous metaplasia present in the mucosa surrounding low-grade MALT lymphoma-like lesions was also histologically confirmed. Similar findings were seen in mice infected with *H. pylori*. In conclusion, this

study described that *H. heilmannii* infection causes severe gastric pathologies and alterations in the expression pattern of gastric mucins, such as Muc6 and Muc13. An infection with this agent also induces a disruption of the gastric homeostasis by causing loss of parietal cells, resulting in the development of mucous metaplasia.

A critical step in gastric *Helicobacter* colonization is adhesion to the gastric mucosa. Bacterial outer membrane proteins (OMPs) are directly involved in the interaction of symbiotic bacteria with their host. *H. heilmannii* is equipped with a large set of OMPs which role in the adhesion process remains to be further elucidated. Interestingly, this bacterium lacks all *H. pylori* adhesins described so far, highlighting that other OMPs are involved in binding. In the **second study** of this thesis, we investigated the role of the *H. heilmannii hof* gene locus encoding the HofH/F/E/G/C/D OMPs in adhesion to the gastric mucosa. This locus is absent in *H. pylori.* Actually, the *hof* genes of *H. pylori* are scattered across its genome. Additionally, the role of the Hof OMPs in gastric MUC13/Muc13 expression during *H. heilmannii* colonization was studied as well. We first defined the *H. heilmannii hof* gene expression patterns in response to different conditions reflecting the gastric environment. Based on these results and the genomic analysis of the *H. heilmannii hof* gene locus, HofE, HofF and HofG were selected for further study. *H. heilmannii ΔhofE*, *ΔhofF* and *ΔhofG* mutants were created and tested *in vitro* and *in vivo* for their adhesion characteristics and their capacity to induce gastric MUC13/Muc13 expression. In *H. pylori* research, only the HofF OMP has been shown to play a role in gastric colonization. Therefore, a *H. pylori ΔhofF* mutant was included for the purpose of comparison. *H. heilmannii* strains lacking HofE or HofF showed a clear decrease in binding to gastric mucins and epithelial cells as well as a lower gastric colonization level in the stomach of Balb/c mice at 4 and 9 weeks post-infection compared to the *H. heilmannii* wildtype strain. The previously shown role of *H. pylori* HofF in gastric colonisation was also confirmed. Interestingly, *H. heilmannii* Δ*hofE* and Δ*hofF* and *H. pylori* Δ*hofF* did not induce an increased expression of MUC13 in human gastric epithelial cells and of Muc13 in the stomach of mice. Finally, we demonstrated that IL-1β is induced in the stomach as a response to *H. pylori* and *H. heilmannii* colonization which on its turn is involved in the expression of MUC13/Muc13 in the gastric epithelium. These novel results in *Helicobacter* research in general identified *H. heilmannii* HofE and HofF as adhesins and suggest an important role of *H. heilmannii* HofE and HofF and *H. pylori* HofF in IL-1β-induced gastric MUC13/Muc13 expression.

In conclusion, research included in this thesis demonstrated that both an acute and chronic *H. heilmannii* infection in mice induce changes in the distribution and expression of mucins in the stomach, in particular Muc6 and Muc13.

Finally, we identified two novel *H. heilmannii* adhesins, HofE and HofF, which also seem to be involved in gastric MUC13 signalling.

SAMENVATTING

Helicobacter pylori is de best bestudeerde *Helicobacter* soort bij de mens. Deze kiem wordt geassocieerd met gastritis, peptische ulcera en maagkanker. De mens kan evenwel ook besmet worden door zogenaamde gastrale niet-*H. pylori Helicobacter* species. Deze kiemsoorten zijn afkomstig van dieren, zoals varkens, honden en katten. *H. heilmannii* is een niet-*H. pylori Helicobacter* species dat de maag van honden en katten koloniseert. Bij de mens veroorzaakt deze kiem gastritis, peptische ulcera en lymfomen van het lymfoïd weefsel van de maagmucosa (MALT-lymfomen). Bovendien is het risico op het ontwikkelen van MALT-lymfomen hoger bij een infectie met niet-*H. pylori* helicobacters dan bij een infectie met *H. pylori*. *H. heilmannii* werd gedetecteerd bij 8-19% van de mensen met maagklachten, veroorzaakt door niet-*H. pylori Helicobacter* species. Dit percentage is waarschijnlijk een onderschatting van de werkelijke prevalentie, aangezien het moeilijk is om een diagnose te stellen van een infectie met deze kiem. Het kan ook niet uitgesloten worden dat een *H. heilmannii* infectie soms zonder symptomen verloopt of enkel milde klachten veroorzaakt die niet grondig onderzocht worden.

Het voorkomen van *H. pylori* infecties in de westerse samenleving is aan het afnemen, wat zou kunnen betekenen dat er meer ruimte gecreëerd wordt voor infecties met niet-*H. pylori* helicobacters. Mensen die in nauw contact leven met honden en katten hebben ook meer kans om besmet te geraken met *H. heilmannii*.

Aanvankelijk was er zeer weinig informatie beschikbaar over de pathogenese van de maagpathologieën veroorzaakt door een infectie met *H. heilmannii*. Dit onderzoek wordt voornamelijk bemoeilijkt doordat het bijzonder moeilijk is om deze kiem te kweken in het laboratorium. De eerste succesvolle *in vitro* isolatie van *H. heilmannii* uit kattenmagen in 2011, opende nieuwe deuren om de rol van dit agens in de humane maagpathologie te kunnen bestuderen.

De maagmucosa wordt bedekt door een mucuslaag die voornamelijk bestaat uit sterk geglycosyleerde mucines en ervoor zorgt dat vastzittende partikels worden weggewassen. Dit is één van de eerste barrières die *Helicobacter* tegenkomt in de maag. Het maagepitheel onder de mucuslaag bestaat uit verschillende celtypes. Een infectie met *H. pylori* kan leiden tot veranderingen in het expressiepatroon en de distributie van maagmucines en tot aantasting van de maagmucosa door verlies van epitheliale cellen. Een verlies van pariëtaalcellen kan leiden tot mucosale metaplasie, wat gekenmerkt wordt door de aanwezigheid van mucines die

in een gezonde maagmucosa normaal niet voorkomen. *H. heilmannii* is, net zoals *H. pylori*, in staat zich vast te hechten aan humane maagmucines en maagepitheelcellen. Beide species bevinden zich voornamelijk dicht tegen het oppervlakte-epitheel, maar *H. heilmannii* kan ook teruggevonden worden in nauw contact met de zuur-secreterende pariëtaalcellen aanwezig in de maagklieren.

Bij aanvang van deze thesis was er nog geen informatie beschikbaar over de dynamiek van mucines in de maag tijdens een infectie met *H. heilmannii.* Het was tevens ook niet gekend hoe deze bacterie bindt aan de maagmucosa, wat een essentiële stap is om de infectie te initiëren en in stand te houden.

Tijdens een **eerste studie** werd het expressiepatroon en de distributie van mucines in de maag van muizen bestudeerd gedurende 1 jaar na een initiële *H. heilmannii* infectie. Tijdens deze dierproef evolueerde de maagpathologie van gastritis tot lesies die sterk lijken op MALT-lymfomen. Merkers voor zuurproductie door pariëtaalcellen en mucosale metaplasie werden ook onderzocht. Een muis-geadapteerde *H. pylori* stam, die ook MALT-lymfomen kan induceren in de maag van muizen, werd mee ingesloten ter vergelijking. Na 9 weken infectie was de mRNA expressie van het Muc6 mucine duidelijk verhoogd, zowel in het antrum als in de fundus van de maag van de met *H. heilmannii*- en *H. pylori*-geïnfecteerde muizen. Vanaf 1 dag na de initiële infectie was de mRNA expressie van Muc13 reeds verhoogd in de fundus van de maag van de met *H. heilmannii*- en *H. pylori*-geïnfecteerde muizen, wat een interessante en nieuwe bevinding is. Het expressieniveau van Muc13 in de maag bleef hoog gedurende de gehele infectieproef. In een gezonde maag komt Muc13 niet tot expressie en tot nu toe werd een verhoogde expressie van dit mucine enkel beschreven bij maagkanker. Daarnaast kon de verhoogde expressie van zowel Muc6 als Muc13 tijdens het acuut stadium van infectie, positief gecorreleerd worden met een toenemend aantal *H. heilmannii* bacteriën in de maag. Dit duidt erop dat deze mucines een rol spelen tijdens de eerste fase van kolonisatie van de maagmucosa door *H. heilmannii*. Bij de met *H. pylori*-geïnfecteerde muizen kon een toenemend aantal *H. pylori* bacteriën in de maag positief gecorreleerd worden met een verhoogde expressie van Muc13, maar niet met een verhoogde expressie van Muc6.

Tijdens latere stadia van de infectie met *H. heilmannii* daalde de mRNA expressie van H^+/K^+ ATPase α/β en KCNQ1 (merkers voor zuurproductie), terwijl de expressie van Muc4, Tff2, Dmbt1 en PigR (merkers voor metaplasie van de mucuslaag) steeg vanaf 16 weken infectie. Dit suggereert de aanwezigheid van metaplasie (“spasmolytic polypeptide-expressing

metaplasia") in de fundus van de maag van met *H. heilmannii*-geïnfecteerde muizen. Ook na histologische analyse kon metaplasie in de maagmucosa rondom de lesies gelijkend op MALT-lymfomen bevestigd worden. Dezelfde resultaten werden teruggevonden in de maag van de muizen geïnfecteerd met *H. pylori*. Als conclusie kunnen we stellen dat een *H. heilmannii* infectie ernstige maagpathologieën veroorzaakt en veranderingen kan teweegbrengen in het expressiepatroon van maagmucines zoals Muc6 en Muc13. Infectie met *H. heilmannii* kan ook de homeostase in de maag verstoren door een verlies aan pariëtaalcellen te induceren, wat aanleiding kan geven tot de ontwikkeling van mucosale metaplasie.

Adhesie aan de maagmucosa is een zeer belangrijke stap in het kolonisatieproces van gastrale helicobacters. Bacteriële buitenste membraanproteïnen zijn rechtstreeks betrokken bij de interactie van symbiotische kiemen met hun gastheer. *H. heilmannii* beschikt over een uitgebreide set van buitenste membraanproteïnen, maar de rol van deze eiwitten in het adhesieproces was bij de aanvang van dit doctoraatsonderzoek niet gekend. Het is opmerkelijk dat alle buitenste membraanproteïnen die bij *H. pylori* functioneren als adhesines, afwezig zijn bij *H. heilmannii*. Dit impliceert dat er andere eiwitten betrokken zijn bij adhesie van *H. heilmannii* aan de maagmucosa. Daarom werd tijdens de **tweede studie** van deze thesis de rol van het *hof* locus van *H. heilmannii*, dat codeert voor de HofH/F/E/G/C/D buitenste membraanproteïnen, in de adhesie van dit agens aan de maagmucosa onderzocht. Dit *hof* locus is afwezig bij *H. pylori*. Bij dit species liggen de *hof* genen verspreid over het hele genoom. Daarnaast werd ook de rol van deze Hof eiwitten in de expressie van MUC13/Muc13 tijdens kolonisatie van de maagmucosa door *H. heilmannii* bestudeerd.
Eerst werden de expressiepatronen van de *hof* genen van *H. heilmannii* bestudeerd als respons op verschillende gesimuleerde maagcondities. Daarnaast werd er ook een genoomanalyse uitgevoerd van het *H. heilmannii hof* locus. Op basis van de bekomen resultaten werden HofE, HofF en HofG geselecteerd voor verdere analyse. Er werden *ΔhofE*, *ΔhofF* en *ΔhofG* mutanten van *H. heilmannii* gecreëerd en zowel hun bindingseigenschappen als hun mogelijkheid om MUC13/Muc13 expressie te induceren in het maagepitheel, werden *in vitro* en *in vivo* getest. Voor *H. pylori* werd er reeds aangetoond dat enkel het HofF eiwit een rol speelt bij de kolonisatie van de maag. In ons onderzoek werd er daarom ook een *ΔhofF* mutant van *H. pylori* ingesloten om vergelijking tussen beide bacteriën toe te laten. In vergelijking met de wild-typestam van *H. heilmannii*, vertoonden de *H. heilmannii* mutanten

zonder HofE of HofF een duidelijk lagere bindingscapaciteit aan maagmucines en epitheelcellen. Beide mutanten hadden ook een lagere kolonisatiecapaciteit in de maag van muizen op 4 en 9 weken na de initiële infectie.. De rol van het HofF eiwit in de kolonisatie van de maag door *H. pylori* werd bevestigd in deze studie. Opmerkelijk was dat *H. heilmannii* Δ*hofE* en Δ*hofF* en *H. pylori* Δ*hofF* geen verhoogde expressie induceerden van MUC13 in humane maagepitheelcellen of van Muc13 in de maag van geïnfecteerde muizen. Tenslotte werd er ook aangetoond dat kolonisatie van de maagmucosa door *H. pylori* en *H. heilmannii* leidt tot inductie van het cytokine IL-1β in de maag, dat op zijn beurt betrokken is bij de expressie van MUC13/Muc13 in het maagepitheel.
Deze vernieuwende resultaten voor het *Helicobacter* onderzoek resulteerden in de identificatie van de *H. heilmannii* HofE en HofF eiwitten als adhesines en tonen een belangrijke rol aan voor *H. heilmannii* HofE en HofF en *H. pylori* HofF in de IL-1β-geïnduceerde expressie van MUC13/Muc13 in het maagepitheel.

Als conclusie kunnen we stellen dat dit doctoraatsonderzoek heeft aangetoond dat zowel een acute als een chronische *H. heilmannii* infectie in muizen veranderingen in de distributie en expressie van maagmucines induceert, met in het bijzonder een verhoogde expressie van Muc6 en Muc13. Er werd eveneens aangetoond dat deze mucines een rol spelen in de kolonisatie van de maag door *H. heilmannii*.
Daarnaast werden twee adhesines geïdentificeerd bij *H. heilmannii*, namelijk HofE en HofF. Deze buitenste membraanproteïnen spelen ook een rol in de MUC13 signalisatie in de maag.

CURRICULUM VITAE

Cheng Liu was born in Urumqi, Xinjiang province, China, on the 07th of November, 1986. She obtained her bachelor degree in veterinary medicine at the National Key Laboratory of Microbiology, Jilin University in Changchun, Jilin province in July, 2009. Thereafter, she started her master study in preventive veterinary medicine at the Agricultural University of Sichuan. Her research topic was the study of the Avian Infectious Bronchitis (IBV) virus S1 protein as a new candidate target for IBV vaccine development. This work was performed under the supervision of Prof. Yong Huong. She completed her master studies in July, 2011.

Soon after, she continued to pursue her doctoral studies at the department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Belgium, under the supervision of Prof. dr. Freddy Haesebrouck and Dr. Ir. Annemieke Smet. Her doctoral research was supported by a grant from the China Scholarship Council (Grant No. 2011691031; September 2011) and by grants from the Research Fund of Ghent University, Belgium (Grant No. GOA 01G00408 and 01SC0312). This PhD research dealt with the identification of bacterial outer membrane proteins and host mucins involved in the colonization of the gastric mucosa by the zoonotic pathogen *Helicobacter heilmannii*. Her research has resulted in several publications in leading journals and her results were also presented at several international academic conferences.

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Cheng Liu